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# UTILITY PATENT APPLICATION TRANSMITTAL

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Attorney Docket No. 98,736

First Named Inventor Roderick L. Halb

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**APPLICATION ELEMENTS**

1.  Transmittal Form with Fee
2.  Specification (including claims and abstract) [Total Pages 76]
3.  Drawings [Total Sheets 41]
4.  Oath or Declaration [Total Pages ]  
 a.  Newly executed  
 b.  Copy from prior application  
**[Note Boxes 5 and 17 below]**  
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8.  Assignment Papers
9.  Power of Attorney
10.  English Translation Document (if applicable)
11.  Information Disclosure Statement (IDS)  
 PTO-1449 Form  
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12.  Preliminary Amendment
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<b>APPLICATION FEES</b>			
<b>BASIC FEE</b>			\$790.00
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Independent Claims	2	- 3=	x \$ 82.00      \$0
<input checked="" type="checkbox"/> Multiple Dependent Claims(s) if applicable			+\$270.00      \$270.00
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**UTILITY PATENT APPLICATION TRANSMITTAL**

Attorney Docket No. 98,736

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Date	Dec. 22, 1998

UTILITY (Rev. 11/18/97)

**Method for Accelerating the Rate of Mucociliary Clearance**

5    **Field of the Invention**

The present invention relates to compositions comprising serine protease inhibitor proteins which stimulate the rate of mucociliary clearance of mucus and sputum in lung airways. The present invention also relates to methods for stimulating the rate of mucociliary clearance in mammals.

10

**Background of the Invention**

***Problem Addressed***

Mucociliary dysfunction, characterized by the inability of ciliated epithelium to clear mucus and sputum in lung airways, is a serious complication of chronic obstructive lung diseases such as Chronic Bronchitis (CB), Bronchiectasis (BE), asthma and, especially, Cystic Fibrosis (CF). Patients suffering from mucociliary dysfunction are particularly vulnerable to secondary bacterial infections. Treatment and maintenance modalities for CF and other respiratory diseases associated with mucociliary dysfunction and the need for improved treatments have been described. See, for instance, Braga "Drugs in Bronchial Mucology, Raven Press, New York, 1989; Lethem et al, Am Rev. Respir. Dis. 142:1053-1058, 1990; U.S. Patent No. 5,830,436..

25    ***Cystic Fibrosis***

Cystic fibrosis (CF) is an autosomal recessive disease that causes abnormalities in fluid and electrolyte transport in exocrine epithelia. Mutations within the DNA coding for a protein termed the cystic fibrosis transmembrane conductance regulator (CFTR) have been found in virtually all CF patients. Cells 30 of the lung are particularly affected. Di Santagrese et al, Am J. Med. 66: 121-132 (1979).

In CF, the luminal border of the airway mucosal cell is unresponsive to cAMP-dependant protein kinase activation of membrane chloride ion channels. The cell permeability to Cl<sup>-</sup> is impaired and Na<sup>+</sup> absorption across the cell 35 membrane is accelerated. Both of these electrolyte imbalances tend to reduce the level of hydration of the airway mucus thus contributing to the viscous lung secretions characteristic of CF. Knowles, Clin. Chest. Med. 11: 75 (1986).

Adventitious bacteria and mycoplasmas enter the lung airways and establish colonies within the mucus. The thick mucus associated with CF isolates these pathogens from the immune system. Since mucociliary clearance is reduced in CF patients, bacterial clearance is also reduced. Lung congestion and infection  
5 are thus common. The prolonged presence of these pathogenic agents invariably initiates inflammatory reactions that compromise lung function.  
Bedrossian et al., Human Pathol. 7:195-204, 1976.

Mucus viscosity in CF lungs is in part due to the decreased hydration of the mucus as related to Cl<sup>-</sup> channel malfunction and modification of sodium  
10 (Na<sup>~</sup>) ion concentration in the airway surface liquid (ASL) that change the rate of airway mucociliary clearance (MCC). The mechanisms involved in mucus transport have been studied in vitro and in vivo. CB, CF, and BE sputa are transported slowly by the mammalian ciliated epithelium of the mucus depleted bovine trachea (MDBT) (Wills et al, J. Clin. Invest. 97(1): 9-13, 1995). Slow  
15 transportability of diseased sputum on the MDBT may be linked to its low electrolyte/osmolyte content (Wills et al, J. Resp. Crit. Care Med. 151(4): 1255-1258,1997). Indeed, diseased sputum is known to have low electrolyte content relative to plasma (Matthews et al, Am. Rev. Resp. Dis. 88: 199-204, 1963 ; Potter et al, Am. Rev. Resp. Dis. 67(1): 83-87,1967 ; Tomkiewicz et al, Am. Rev. Resp. Dis. 148(4, Pt. 1): 1002-1007, 1993).

Further studies on the MDBT have shown that transportability of diseased mucus is markedly improved following treatment with sodium chloride (Wills et al 1995). Furthermore, clinical studies have shown that inhalation of hypertonic saline, or of the epithelial sodium channel (ENaC)  
25 blocker amiloride can significantly increase MCC in diseased patients (Robinson et al , Thorax 52(10): 900-903, 1997; App et al, Am. Rev. Resp. Dis. 141, 605-612, 1990). Recently, the relationship between mucus clearance and its ionic composition in vivo in the guinea-pig model of tracheal mucus velocity (TMV) has been elucidated. In vivo studies showed that a 5 minute aerosol of  
30 hypertonic saline transiently increased TMV. An increase in TMV was observed 1 min after hypertonic saline (14.4%) aerosol. TMV was  $5.1 \pm 1.0 \text{ mm} \cdot \text{min}^{-1}$  (n=9) in 0.9% saline-exposed animals compared to  $11.3 \pm 1.3 \text{ mm} \cdot \text{min}^{-1}$  in hypertonic saline exposed animals (n=9; p≤0.001)(Newton & Hall, 1997). Inhaled amiloride also caused an increase in TMV. A significant increase in TMV was observed 15 minutes after a 20 minute aerosol of amiloride (10mM). TMV was  $3.2 \pm 2.5 \text{ mm} \cdot \text{min}^{-1}$  (n=9) in water-exposed animals compared to  $8.1 \pm 0.3 \text{ mm} \cdot \text{min}^{-1}$  in amiloride-exposed animals (n=8; p≤0.05) Newton et al, Ped. Pulm. S17, Abs. 364,

1998). These agents would appear to act by increasing the ionic content of airway surface liquid (ASL).

Recently, a serine protease termed channel activating protease-1 (CAP-1) has been found in the apical membrane of amphibian *Xenopus* kidney epithelial 5 cells (A6 cells) (Vallet et al, *Nature* 389(6651): 607-610, 1997). CAP-1 appears to modulate Na<sup>+</sup> channel activity in these cells. Exposure of the apical membrane to the prototypical bovine Kunitz inhibitor, aprotinin, reduced transepithelial Na<sup>+</sup> transport (Vallet et al 1997 : Chraibi et al, *J. Gen. Physio.* 111(1): 127-138, 1998). The effect of Bikunin, a two Kunitz domain human homologue of bovine 10 aprotinin (Delaria et al, *J. Biol. Chem.* 272(18): 12209-12214, 1997; Marlor et al, *J. Biol. Chem.* 272(18): 12202-12208, 1997), was evaluated using normal cultured human bronchial epithelial cell (HBE) short circuit current (Isc) in vitro (McAulay et al, *Ped. Pulm. S17*, Abs. 141, 1998). Bikunin (1.5ug.ml<sup>-1</sup>: 70nM) significantly inhibited 54% Na<sup>+</sup> Isc in normal HBE cells (n=5-8 ; p≤0.05). Overall, 15 Bikunin (70nM) inhibited 58% of the baseline Isc in 90 minutes. In a further study, Bikunin (5ug.ml<sup>-1</sup>) significantly inhibited 84% Na<sup>+</sup> Isc in normal HBE cells (n=6; p≤0.01) whilst the serpin-family serine protease inhibitor alpha(1)-protease inhibitor ( $\alpha_1$ -PI)(50 ug.ml<sup>-1</sup>) was without a significant effect.

Two recent studies by a single research group have demonstrated a 20 protease inhibitor induced effect on TMV.  $\alpha_1$ -PI (10mg) given either 30 min before antigen challenge, or 1 h after challenge, attenuated antigen-induced reduction in TMV in allergic sheep, 6h after challenge (O'Riordan et al, *Am. J. Resp. Crit. Care Med.* 97(5): 1522-1528, 1997). In Fig 1 in the O'Riordan et al 1997 paper, the authors showed that  $\alpha_1$ -PI administered on its own (no antigen 25 challenge) to the airways of allergic sheep, had no effect on baseline TMV over a 6h period. In the second study,  $\alpha_1$ -PI was given 6 h after antigen challenge and caused only a significant reversal of the antigen-induced fall in TMV at 24 h after challenge (O'Riordan et al, *J. App. Physio.* 85(3): 1086-1091, 1998). The authors argue that the mechanism for the effect of  $\alpha_1$ -PI is associated with its anti- 30 neutrophil elastase property, where neutrophil elastase is believed to be the enzyme responsible for the reduced rate of mucociliary clearance in their model. They reasoned that  $\alpha_1$ -PI could be used to treat mucociliary dysfunction brought about by allergy-induced neutrophil elastase release in asthma (O'Riordan et al 1998); they did not speculate on a potential role in other respiratory diseases.

## Brief Summary of the Invention

The instant invention is directed to the use of Kunitz-family serine protease inhibitors that stimulate the rate of mucociliary clearance (MCC) of mucus and sputum in the airways of the lung. Kunitz-serine protease inhibitors  
5 could be used to treat lung diseases such as Cystic Fibrosis (CF), Chronic Bronchitis (CB) and Bronchiectasis (BE) where the retention and accumulation of mucus is a major clinical problem. Until now, prior art has not associated protease inhibitors with the ability to increase the rate of MCC above baseline rate. Kunitz-type serine protease inhibitors could also be used to treat chronic  
10 sinusitis and glue ear where the retention and accumulation of mucus is a clinical problem.

The instant invention contemplates the use of serine protease inhibitors proteins which include Kunitz domains or Kunitz-like domains for use in a method for stimulating MCC. In one embodiment of the invention, bovine serine protease inhibitor proteins such as aprotinin and variants and fragments thereof such as the ones described in EP 821007, published January 28, 1998 may be used in practicing the invention.  
15

In another embodiment of the invention, human serine protease inhibitors are contemplated for use in the method for stimulating the rate of MCC.  
20 Representative examples of human serine protease inhibitors include Bikunin and variants and fragments thereof such as the ones described in WO 97/33996, published September 18, 1997 (Bayer Corp.), and U.S. Patent No. 5,407,915, issued April 18, 1995 (Bayer AG) which are incorporated herein in its entirety.

## Description of the Drawings

25 The invention will be better understood from a consideration of the following detailed description and claims, taken in conjunction with the drawings, in which:

Figure 1 depicts the nucleotide sequence of EST R35464 (SEQ ID NO.: 12) and the translation of this DNA sequence (SEQ ID NO.: 13) which yielded an  
30 open reading frame with some sequence similarity to aprotinin. The translation product contains 5 of the 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). The position normally occupied by the remaining cysteine (at codon 38) contained instead a phenylalanine (indicated by an asterisk).

35 Figure 2 depicts the nucleotide sequence of EST R74593 (SEQ ID NO.: 14), and the translation of this DNA sequence (SEQ ID NO.: 15) which yielded an open reading frame with homology to the Kunitz class of serine protease

inhibitor domains. The translation product contained 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). However, this reading frame sequence includes stop codons at codon 3 and 23.

5       Figure 3 depicts a deduced nucleic acid sequence of human placental bikunin (SEQ ID NO.: 9) labeled "consensus" and matched with the translated protein amino acid sequence labeled "translated" (SEQ ID NO.: 10). Also as comparison are shown the nucleic acid sequence for ESTs H94519 (SEQ ID NO.: 16), N39798 (SEQ ID NO.: 17), R74593 (SEQ ID NO.: 14) and R35464 (SEQ ID  
10 NO.: 12). The underlined nucleotides in the consensus sequence correspond to the site of PCR primers described in the Examples. Underlined amino acids in the translated consensus sequence are residues whose identity have been confirmed by amino acid sequencing of purified native human placental bikunin. Nucleotide and amino acid code are standard single letter code, "N" in  
15 the nucleic acid code indicates an unassigned nucleic acid, and "\*" indicates a stop codon in the amino acid sequence.

20       Figure 4A depicts the original overlay of a series of ESTs with some nucleic acid sequence homology to ESTs encoding human placental bikunin, or portions thereof. Shown for reference are the relative positions of bikunin (7-64) and bikunin (102-159), labeled KID1 and KID2 respectively.

25       Figure 4B depicts a subsequent more comprehensive EST overlay incorporating additional ESTs. Numbers on the upper X-axis refer to length in base pairs, starting at the first base from the most 5' EST sequence. The length of each bar is in proportion to the length in base pairs of the individual ESTs including gaps. The EST accession numbers are indicated to the right of their respective EST bars.

30       Figure 4C depicts the corresponding alignment of the oligonucleotide sequences of each of the overlapping ESTs shown schematically in Figure 4B. The upper sequence (SEQ ID NO.: 51) labeled bikunin represents the consensus oligonucleotide sequence derived from the overlapping nucleotides at each position. The numbers refer to base-pair position within the EST map. The oligonucleotides in EST R74593 that are bold underlined (at map positions 994 and 1005) are base insertions observed in R74593 that were consistently absent in each of the other overlapping ESTs.

35       Figure 4D depicts the amino acid translation of the consensus oligonucleotide sequence for bikunin depicted in Figure 4C (SEQ ID NO.: 45).

Figure 4E depicts the nucleotide sequence (SEQ ID NO.: 46) and

corresponding amino acid translation (SEQ ID NO.: 47) of a placental bikunin encoding sequence that was derived from a human placental cDNA library by PCR-based amplification.

Figure 4F depicts the nucleotide sequence (SEQ ID NO.: 48) and  
5 corresponding amino acid translation (SEQ ID NO.: 49) of a native human  
placental bikunin encoding clone that was isolated from a human placental  
lambda cDNA library by colony hybridization.

Figure 4G compares the alignment of the amino acid translated  
oligonucleotide sequences for placental bikunin obtained by EST overlay (SEQ  
10 ID NO.: 45), PCR based cloning (SEQ ID NO.: 47), and conventional lambda  
colony hybridization (SEQ ID NO.: 49).

Figure 5 shows a graph of purification of human placental bikunin from placental  
tissue after Superdex 75 Gel-Filtration. The plot is an overlay of the protein elution  
profile as measured by OD 280 nm (solid line), activity of eluted protein in a trypsin  
15 inhibition assay (% inhibition shown by circles), and activity of eluted protein in a  
kallikrein inhibition assay (% inhibition shown by squares).

Figure 6 shows a graph which plots the purification of human placental bikunin  
from placental tissue using C18 Reverse-Phase Chromatography. The plot is an overlay  
of the protein elution profile as measured by OD 215 nm (solid line), activity of eluted  
protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of  
20 eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 7 depicts a silver stained SDS-PAGE gel of highly purified  
placental bikunin (lane 2), and a series of molecular size marker proteins (lane 1)  
of the indicated sizes in kilodaltons. Migration was from top to bottom.

Figure 8 shows the amount of trypsin inhibitory activity present in the  
cell-free fermentation broth from the growth of yeast strains SC101 (panel 8A) or  
WHL341 (panel 8B) that were stably transformed with a plasmid (pS604) that  
directs the expression of placental bikunin (102-159).

Figure 9 shows both a silver stained SDS-PAGE (left panel) and a Western  
30 blot with anti-placental bikunin (102-159) pAb (right panel) of cell-free  
fermentation broth from the growth of yeast strain SC101 (recombinants 2.4 and  
2.5) that was stably transformed with a plasmid directing the expression of  
either bovine aprotinin, or placental bikunin (102-159). Migration was from top  
to bottom.

Figure 10 is a photograph which shows a silver stained SDS-PAGE of  
35 highly purified placental bikunin (102-159) (lane 2) and a series of molecular size  
marker proteins (lane 1) of the indicated sizes in Kilodaltons. Migration was

from top to bottom.

Figure 11 is a photograph which shows the results of Northern blots of mRNA from various human tissues that was hybridized to a  $^{32}\text{P}$  labeled cDNA probe encoding either placental bikunin (102-159) (panel 11A) or encoding placental bikunin (1-213) (panel 11B). Migration was from top to bottom. The numbers to the right of each blot refer to the size in kilobases of the adjacent RNA markers. The organs from which mRNA was derived is described under each lane of the blot.

Figure 12 depicts an immunoblot of placental derived placental bikunin with rabbit antiserum raised against either synthetic reduced placental bikunin (7-64) (panel A) or 102-159 (panel B). For each panel, contents were: molecular size markers (lanes 1); native placental bikunin isolated from human placenta (lanes 2); synthetic placental bikunin (7-64) (lanes 3) and synthetic placental bikunin (102-159) (lanes 4). Tricine 10-20% SDS-PAGE gels were blotted and developed with protein A-purified primary polyclonal antibody (8  $\mu\text{g}$  IgG in 20 ml 0.1% BSA/Tris-buffered saline (pH 7.5), followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Migration was from top to bottom.

Figure 13 depicts a Coomassie Blue stained 10-20% Tricine SDS-PAGE gel of 3 micrograms of highly purified placental bikunin (1-170) derived from a baculovirus / Sf9 expression system (lane 2). Lane 1 contains molecular size markers. Migration was from top to bottom.

Figure 14 depicts a comparison of the effect of increasing concentrations of either Sf9-derived human placental bikunin (1-170) (filled circles), synthetic placental bikunin (102-159) (open circles), or aprotinin (open squares) on the activated partial thromboplastin time of human plasma. Clotting was initiated with  $\text{CaCl}_2$ . The concentration of proteins are plotted versus the -fold prolongation in clotting time. The uninhibited clotting time was 30.8 seconds.

Figure 15 illustrates the effect of Bikunin at dosage levels of 2  $\mu\text{M}$  and 0.2  $\mu\text{M}$  relative to amiloride (100  $\mu\text{M}$ ) and Hank's Balanced salt solution (HBSS) vehicle (control) on potential differences in guinea pig trachea 3 hours post-treatment.

Figure 16 illustrates (a) the postioning of the instillment syringe and beta probe relative to the guinea pig trachea; (b) a representative graph for measurement of trachea mean velocity (TMV) using  $^{32}\text{P}$ -labelled *S.cerevisiae*; and (c) the sustained increase in TMV in vivo in guinea pig in response to Bikunin (5  $\mu\text{g}$ ) relative to HBSS vehicle control at 1.5, 1.75, 2.0, 2.25 and 2.5 hours following tracheal instillment.

Figure 17 illustrates that Bikunin (70 nM)decreases sodium current in cultured human bronchial epithelial cells in vitro relative to amiloride (10  $\mu\text{M}$ ).

Figure 18 illustrates the effect of a 5 min aerosol of hypertonic saline (14.4%) on increasing TMV, following aerosol treatment in guinea pig trachea.

Figure 19 illustrates the effect of a 20 minute aerosol of amiloride (10 mM) on TMV, following aerosol treatment in guinea pig trachea.

5 Figure 20 illustrates that Bikunin (5ug/mL), aproteininin (5 ug/mL), and aprotinin double mutein (0.5 ug/mL, 1.5 ug/mL and 5 ug/mL) decrease sodium short circuit current in cultured human bronchial cells in vitro.

### Detailed Description of the Invention

10 The present invention relates to compositions comprising Kunitz-type serine protease inhibitor proteins and fragments thereof which stimulate the rate of mucociliary clearance of mucus and sputum in lung airways. The compositions also encompass a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class.

15 The present invention also provides methods for stimulating the rate of mucociliary clearance in patients with mucociliary dysfunction, wherein an effective amount of the disclosed serine protease inhibitors of the present invention, in a biologically compatible vehicle, is administered to the patient.

20 A preferred application for placental bikunin, isolated domains, and other variants is for stimulating mucociliary clearance in CF patients as part of disease therapy and management. These methods and compositions reduce or eliminate mucus and sputum buildup in lung airways in patients with chronic obstructive lung disease, thereby reducing the risk of secondary lung infections and other 25 adverse side effects , as well as avoiding or delaying the need for lung transplant surgery in CF patients.

25 The method of the present invention contemplates the use of aprotinin to stimulating MCC. Aprotinin has been shown to reduce transepithelial Na<sup>+</sup> transport in the apical membrane of amphibian *Xenopus* kidney epithelial cells (A6 cells) (Vallet et al 1997 : Chraibi et al 1998). The mechanism of aprotinin action has been proposed to involve inhibition of CAP-1, a protease involved in modulating Na<sup>+</sup> channel activity in A6 cells. Bikunin, a two Kunitz domain human homologue of bovine aprotinin (Delaria et al 1997 : Marlor et al 1997), was also shown to significantly inhibit normal cultured human bronchial 30 epithelial cell (HBE) short circuit current (Isc) in vitro (McAulay et al 1998). Bikunin (1.5ug.ml<sup>-1</sup> : 70nM) significantly inhibited 54% Na<sup>+</sup> Isc in normal HBE cells (n=5-8 ; p≤0.05). Overall, Bikunin (70nM) inhibited 58% of the baseline Isc

in 90 minutes. In a further study, Bikunin ( $5\text{ug.ml}^{-1}$ ) significantly inhibited 84%  $\text{Na}^+ \text{Isc}$  in normal HBE cells ( $n=6$ ;  $p\leq 0.01$ ) whilst the serpin-family serine protease inhibitor alpha(1)-protease inhibitor ( $\alpha_1\text{-PI}$ ) ( $50\text{ug.mL}^{-1}$ ) was without a significant effect.

5       In light of these observations, Kunitz-type serine inhibitors such as aprotinin, placental bikunin and fragments thereof are contemplated as therapeutics for treating mucociliary dysfunction, including cystic fibrosis.

10      A significant advantage of the Kunitz domains of the serine protease inhibitor Bikunin and fragments and analogs thereof of the present invention is that they are human proteins, and also less positively charged than Trasylol<sup>®</sup> (Example 1), thereby reducing the risk of kidney damage on administration of large doses of the proteins. Being of human origin, the protein of the instant invention can thus be administered to human patients with significantly reduced risk of undesired immunological reactions as compared to administration of similar doses of Trasylol<sup>®</sup>. Furthermore, it was found that bikunin(102-159), bikunin(7-64), and bikunin(1-170) are significantly more potent inhibitors of plasma kallikrein than Trasylol<sup>®</sup> *in vitro* (Example 3, 4 and 10). Thus bikunin and fragments thereof are expected to be more effective *in vivo* relative to aprotinin.

20      The amount of the pharmaceutical composition to be employed will depend on the recipient and the condition being treated. The requisite amount may be determined without undue experimentation by protocols known to those skilled in the art. Alternatively, the requisite amount may be calculated, based on a determination of the amount of target protease such as plasmin, 25 kallikrein or prostasin which must be inhibited in order to treat the condition. As the active materials contemplated in this invention are deemed to be nontoxic, treatment preferably involves administration of an excess of the optimally required amount of active agent.

30      For stimulating the rates of mucociliary clearance in patients with chronic obstructive lung disease, the proteins of the instant invention may be used like aprotinin Trasylol<sup>®</sup> while taking into account the differences in potency. The use of Trasylol<sup>®</sup> is outlined in the Physicians Desk Reference, 1995, listing for Trasylol<sup>®</sup> supplement A. Briefly, with the patient in a supine position, the loading dose of placental bikunin, isolated domain or other variant is given by infusion slowly over about 20 to 30 minutes. In general, a total dose of between 35 about  $2\times 10^6$  KIU (kallikrein inhibitory units) and  $8\times 10^6$  KIU will be used, depending on such factors as patient weight and condition. Preferred loading

doses are those that contain a total of 1 to 2 million kallikrein inhibitory units (KIU).

The proteins of the instant invention are employed in pharmaceutical compositions formulated in the manner known to the art. Such compositions 5 contain active ingredient(s) plus one or more pharmaceutically acceptable carriers, diluents, fillers, binders, and other excipients, depending on the administration mode and dosage form contemplated. Examples of therapeutically inert inorganic or organic carriers known to those skilled in the art include, but are not limited to, lactose, corn starch or derivatives thereof, talc, 10 vegetable oils, waxes, fats, polyols such as polyethylene glycol, water, saccharose, alcohols, glycerin and the like. Various preservatives, emulsifiers, dispersants, flavorants, wetting agents, antioxidants, sweeteners, colorants, stabilizers, salts, buffers and the like can also be added, as required to assist in the stabilization of the formulation or to assist in increasing bioavailability of the 15 active ingredient(s) or to yield a formulation of acceptable flavor or odor in the case of oral, nasal or pulmonary dosing. The inhibitor employed in such compositions may be in the form of the original compound itself, or optionally, in the form of a pharmaceutically acceptable salt. The compositions so formulated are selected as needed for administration of the inhibitor by any 20 suitable mode known to those skilled in the art.

Parenteral administration modes include intravenous (*i.v.*), subcutaneous (s.c.), intraperitoneal (*i.p.*), and intramuscular (*i.m.*) routes. Intravenous administration can be used to obtain acute regulation of peak plasma concentrations of the drug as might be needed. Alternatively, the drug can be 25 administered at a desired rate continuously by i.v. catheter. Suitable vehicles include sterile, non-pyrogenic aqueous diluents, such as sterile water for injection, sterile-buffered solutions or sterile saline. The resulting composition is administered to the patient prior to and/or during surgery by intravenous injection or infusion.

Improved half life and targeting of the drug to phagosomes such as neutrophils and macrophage involved in inflammation may be aided by entrainment of the drug in liposomes. It should be possible to improve the selectivity of liposomal targeting by incorporating into the outside of the liposomes ligands that bind to macromolecules specific to target organs/tissues 30 such as the GI tract and lungs. Alternatively, *i.m.* or *s.c.* deposit injection with or without encapsulation of the drug into degradable microspheres (e.g., comprising poly-DL-lactide-co-glycolide) or protective formulations containing

- collagen can be used to obtain prolonged sustained drug release. For improved convenience of the dosage form it is possible to use an i.p. implanted reservoir and septum such as the percuseal system. Improved convenience and patient compliance may also be achieved by use of either injector pens (e.g., the Novo
- 5 Pin or Q-pen) or needle-free jet injectors (e.g., from Bioject, Medijet or Becton Dickinson). Precisely controlled release can also be achieved using implantable pumps with delivery to the desired site via a cannula. Examples include the subcutaneously implanted osmotic pumps available from ALZA such as the ALZET osmotic pump.
- 10 Oral delivery may be achieved by incorporating the drug into tablets, coated tablets, dragées, hard and soft gelatin capsules, solutions, emulsions, suspensions or enteric coated capsules designed to release the drug into the colon where digestive protease activity is low. Examples of the latter include the OROS-CT/Osmet<sup>TM</sup> system of ALZA, and the PULSINCAP<sup>TM</sup> system of Scherer Drug Delivery Systems. Other systems use azo-crosslinked polymers that are degraded by colon-specific bacterial azoreductases, or pH sensitive polyacrylate polymers that are activated by the rise in pH in the colon. The above systems may be used in conjunction with a wide range of available absorption enhancers.
- 15 Rectal delivery may be achieved by incorporating the drug into suppositories.
- 20 Nasal delivery may be achieved by incorporating the drug into bioadhesive particulate carriers (<200 nm) such as those comprising cellulose, polyacrylate or polycarbophil, in conjunction with suitable absorption enhancers such as phospholipids or acylcarnitines. Commercially available systems include those developed by Dan Biosys and Scios Nova.
- 25 For stimulating the rate of mucociliary clearance, the preferred mode of administration of the placental bikunin variants of the present invention is pulmonary delivery. The Kunitz-type serine protease inhibitors disclosed herein may be administered to the lungs of a subject by any suitable means, but are preferably administered by administering an aerosol suspension of respirable
- 30 particles comprised of the active compound, which the subject inhales. The respirable particles may be liquid or solid. Micron-sized dry powders containing the medicament in a suitable carrier such as mannitol, sucrose or lactose may be delivered to the lung airway surface using dry powder inhalers such as those of Inhale<sup>TM</sup>, Dura<sup>TM</sup>, Fisons (Spinhaler<sup>TM</sup>), and Glaxo (Rotahaler<sup>TM</sup>), or Astra
- 35 (Turbohaler<sup>TM</sup>) propellant based metered dose inhalers. Solution formulations with or without liposomes may be delivered using nebulizers.

Aerosols of liquid particles comprising the proteins may be produced by

any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer. See, e.g., U.S. Pat. No. 4,501,729. Nebulizers are commercially available devices which transform solutions or suspensions of the active ingredient into a therapeutic aerosol mist either by means of acceleration 5 of compressed gas, typically air or oxygen, through a narrow venturi orifice or by means of ultrasonic agitation. Suitable formulations for use in nebulizers consist of the active ingredient in a liquid carrier. The carrier is typically water (and most preferably sterile, pyrogen-free water) or a dilute aqueous alcoholic solution, preferably made isotonic with body fluids by the addition of, for 10 example, sodium chloride. Optional additives include preservatives if the formulation is not made sterile, for example, methyl hydroxybenzoate, antioxidants, flavoring agents, volatile oils, buffering agents and surfactants.

Aerosols of solid particles comprising the protein may likewise be produced with any solid particulate medicament aerosol generator. Aerosol 15 generators for administering solid particulate medicaments to a subject produce particles which are respirable, as explained above, and generate a volume of aerosol containing a predetermined metered dose of a medicament at a rate suitable for human administration. One illustrative type of solid particulate aerosol generator is an insufflator. Suitable formulations for administration by 20 insufflation include finely comminuted powders which may be delivered by means of an insufflator or taken into the nasal cavity in the manner of a snuff. In the insufflator, the powder (e.g., a metered dose thereof effective to carry out the treatments described herein) is contained in capsules or cartridges, typically made of gelatin or plastic, which are either pierced or opened in situ and the 25 powder delivered by air drawn through the device upon inhalation or by means of a manually-operated pump. The powder employed in the insufflator consists either solely of the protein or of a powder blend comprising the protein, a suitable powder diluent, such as lactose, and an optional surfactant. A second type of illustrative aerosol generator comprises a metered dose inhaler. Metered 30 dose inhalers are pressurized aerosol dispensers, typically containing a suspension or solution formulation of the active ingredient in a liquified propellant. During use these devices discharge the formulation through a valve adapted to deliver a metered volume, typically from 10 to 200 uL, to produce a fine particle spray containing the protein. Suitable propellants include certain 35 chlorofluorocarbon compounds, for example, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane and mixtures thereof. The formulation may additionally contain one or more co-solvents, for example,

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ethanol, surfactants, such as oleic acid or sorbitan trioleate, antioxidants and suitable flavoring agents.

For metered dose inhaler or dry powder inhaler devices, the aerosol, whether formed from solid or liquid particles, may be produced by the aerosol generator at a rate of from about 5 to 150 liters per minute, more preferably from about 10 to 100 liters per minute, and most preferably for metered dose inhalers from about 10 to 50 liters per minute, and most preferably for dry powder inhalers about 60 liters per minute. Aerosols generated by nebulizer, jet or ultrasonic, may be produced by the aerosol generator at a rate of from about 1 to 100 liters per minute, more preferably from about 4 to 10 liters per minute. Aerosols containing greater amounts of protein may be administered more rapidly.

The dosage of the protease inhibitor will vary depending on the condition being treated and the state of the subject. The daily dose may be divided among one or several unit dose administrations. The daily dose by weight may range from about 0.1 to 20 milligrams of respirable particles for a human subject, depending upon the age and condition of the subject.

Solid or liquid particulate pharmaceutical formulations containing protease inhibitors of the present invention should include particles of respirable size: that is, particles of a size sufficiently small to pass through the mouth and larynx upon inhalation and into the bronchi and alveoli of the lungs. In general, particles ranging from about 1 to 8 microns in size (more particularly, less than about 6 microns in size) are respirable. Particles of non-respirable size which are included in the aerosol tend to be deposited in the throat and swallowed, and the quantity of non-respirable particles in the aerosol is preferably minimized. For nasal administration, a particle size in the range of 10-500 microns is preferred to ensure retention in the nasal cavity.

In the manufacture of a formulation according to the invention, the protease inhibitor is typically admixed with, *inter alia*, an acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier may be a solid or a liquid, or both, and is preferably formulated with the compound as a unit-dose formulation, for example, a capsule, which may contain from 0.5% to 99% by weight of the active compound. One or more active compounds may be incorporated in the formulations of the invention, which formulations may be prepared by any of the well-known techniques of pharmacy consisting essentially of admixing the components.

Compositions containing respirable dry particles of protease inhibitor may be prepared by grinding the inhibitor with a mortar and pestle, and then passing the micronized composition through a 400 mesh screen to break up or separate out large agglomerates.

5 The pharmaceutical composition may optionally contain a dispersant which serves to facilitate the formation of an aerosol. A suitable dispersant is lactose, which may be blended with the active agent in any suitable ratio (e.g., a 1 to 1 ratio by weight).

If desired, general ex vivo and in vivo gene therapy strategies may 10 employed to deliver nucleic acid constructs encoding Kunitz-type serine protease inhibitor proteins such as Bikunin, Aprotinin or fragments and variants thereof such as the ones described in WO 97/33996 (Bayer Corp.) and U.S. Patent No. 5,407,915.(Bayer AG). Gene therapy techniques that are primarily virus-based have been used to transform pulmonary cells as a means for treating 15 the manifestations of CF in the lung and associated extrapulmonary tissues. See WO 93/03709, published March 3, 1993 which describes the use of retroviral and non-retroviral vectors (e.g., adenoviruses and adeno-associated viruses) for the stable expression of the CFTR gene in CF patients. Alternatively, non-viral methods for delivery of exogenous nucleic acids are also known and are 20 contemplated for use in the instant invention. See WO 93/12240, published June 24, 1993 and references cited therein, describing a transcription or expression cassettes including the coding sequence for a CFTR molecule operably joined to regulatory sequences functional in a mammal. The nucleic acids constructs are then supplied to the airways and alveoli of the lung in a number of ways 25 including aerosolized delivery alone or in combination with lipid-based complexes, e.g., Lipofectin.<sup>TM</sup> WO 95/26356, published October 5, 1995 describes representative examples of lipids useful for transfection. It is therefore contemplated in the instant invention that nucleic acid molecules encoding Kunitz-type serine protease inhibitors such as Bikunin, Aprotinin or variants 30 and fragments thereof may be similarly administered to lung airways by any suitable gene therapy method as a means for stimulating the rate of mucociliary clearance of mucus and sputum in a subject in need of such treatment.

#### Searching Human Sequence Data

35 The existence of a distinct human protein homologous in function to aprotinin, was deduced following a unique analysis of sequence entries to the expressed-sequence-tag data-base (hereafter termed dbEST) at the NCBI

(National Center for Biological Information, Maryland). Using the TBLastN algorithm (BLAST, or Basic Local Alignment Search Tool uses the method of Altschul et a., (1990) J. Mol Biol 215, 00 403-410, to search for similarities between a query sequence and all the sequences in a data-base, protein or nucleic acid in any combination), the data-base was examined for nucleotide sequences bearing homology to the sequence of bovine pre-pro-aprotinin, Trasylol®. This search of numerous clones was selectively narrowed to two particular clones which could possibly encode for a deduced amino acid sequence that would correspond to a human protein homologous in function to aprotinin. The selected nucleic acid sequences were R35464 (SEQ ID NO: 12) and R74593 (SEQ ID NO: 14) that were generated from a human placental nucleic acid library. The translated protein sequence in the longest open reading frame for R35464 (SEQ ID NO: 13) was missing one of the 6 cysteines that are critical for formation of the Kunitz-domain covalent structure, meaning that the nucleic acid sequence of R35464 could not yield a functional inhibitor. Similarly, the longest translated open reading frame from clone R74593 (SEQ ID NO: 15) contained a stop codon 5' to the region encoding the Kunitz like sequence, meaning that this sequence, could not be translated to yield a functional secreted Kunitz domain. The significance of these sequences alone was unclear. It was possible that they represented a) the products of pseudogenes, b) regions of untranslated mRNA, or c) the products of viable mRNA which had been sequenced incorrectly.

### Discovery of Human Bikunin

To specifically isolate and determine the actual human sequence, cDNA primers were designed to be capable of hybridizing to sequences located 5' and 3' to the segment of cDNA encoding our proposed Kunitz like sequences found within R35464 and R74593. The primers used to amplify a fragment encoding the Kunitz like sequence of R74593 were

CGAAGCTTCATCTCCGAAGCTCCAGACG (the 3' primer with a HindIII site; SEQ ID NO.:33) and AGGATCTAGACAATAATTACCTGACCAAGGA (the 5' primer with an XbaI site; SEQ ID NO.:34).

These primers were used to amplify by PCR (30 cycles) a 500 base pair product from a human placental cDNA library from Clontech (MATCHMAKER, Cat #HL4003AB, Clontech Laboratories, Palo Alto, CA), which was subcloned into Bluescript-SK+ and sequenced with the T3 primer with a Sequenase™ kit version 2.0. Surprisingly, the sequence of the fragment obtained using our primers was different from the sequence listed in the dbEST data base for clone

R74593. In particular, our new sequence contained an additional guanosine base inserted 3' to the putative stop codon, but 5' to the segment encoding the Kunitz-like sequence (Figure 3). The insertion of an additional G shifted the stop codon out of the reading frame for the Kunitz-like domain (G at base pair 114 of the 5 corrected sequence for R74593; Figure 3).

Subsequent query of the dbEST for sequences homologous to the Kunitz-like peptide sequence of R74593 yielded H94519 derived from human retina library and N39798. These sequences contained a Kunitz-like sequence that was almost identical to the Kunitz-like domain encoded in R35464 except that it 10 contained all six of the characteristic cysteines. Overlay of each of the nucleotide sequences with that of R74593 (corrected by the insertion of G at b,p, 114) and R35464 was used to obtain a consensus nucleotide sequence for a partial human placental bikunin (SEQ ID NO.: 9; Figure 3). The translated consensus sequence yielded an open reading frame extending from residue -18 to +179 (Figure 3; full 15 translation SEQ ID NO.: 10) that contained two complete Kunitz-like domain sequences, within the region of amino acid residues 17-64 and 102-159 respectively.

Further efforts attempted to obtain additional 5' sequence by querying dbEST with the sequence of R35464. Possible matches from such searches, that 20 possessed additional 5' sequence were then in turn used to re-query the dbEST. In such an iterative fashion, a series of overlapping 5' sequences were identified which included clones H16866, T66058, R34808, R87894, N40851 and N39876 (Figure 4). Alignment of some of these sequences suggested the presence of a 5' ATG which might serve as a start site for synthesis of the consensus translated 25 protein sequence. From this selected information, it was now possible to selectively screen for, and determine the nucleic acid and polypeptide sequences of a human protein with homologous function to aprotinin.

Re-interrogation of the dbEST revealed a number of new EST entries shown schematically in Figure 4B. Overlap with these additional ESTs allowed 30 us to construct a much longer consensus oligonucleotide sequence (Figure 4C) that extended both 5' and 3' beyond the original oligonucleotide sequence depicted in Figure 3. In fact, the new sequence of total length 1.6 kilobases extended all the way to the 3' poly-A tail. The increased number of overlapping ESTs at each base-pair position along the sequence improved the level of 35 confidence in certain regions such as the sequence overlapping with the 3' end of EST R74593 (Figure 3). Several overlapping ESTs in this region corroborated two critical base deletions relative to R74593 (located as bold underlined in Figure

4C, map positions 994 and 1005). Translation of the new consensus sequence (Figure 4D) in the bikunin encoding frame yielded a form of placental bikunin that was larger (248 amino acids) than the mature sequence (179 amino acids) encoded from the original consensus (SEQ ID NO.: 1), and was terminated by an  
5 in-frame stop codon within the oligonucleotide consensus. The size increase was due to a frame shift in the 3' coding region resulting from removal of the two base insertions unique to EST R74593. The frame shift moved the stop codon of the original consensus (Figure 3) out of frame enabling read through into a new frame encoding the additional amino acid sequence. The new translation  
10 product (Figure 4D) was identical to the original protein consensus sequence (SEQ ID NO.: 1) between residues +1 to +175 (encoding the Kunitz domains), but contained a new C-terminal extension exhibiting a putative 24 residue long transmembrane domain (underlined in Figure 4D) followed by a short 31 residue cytoplasmic domain. The precise sequence around the initiator  
15 methionine and signal peptide was somewhat tentative due to considerable heterogeneity amongst the overlapping ESTs in this region.

Analysis of the protein sequence by Geneworks™, highlighted asparagine residues at positions 30 and 67 as consensus sites for putative N-linked glycosylation. Asparagine 30 was not observed during N-terminal  
20 sequencing of the full length protein isolated from human placenta, consistent with it being glycosylated.

### Cloning of Human Bikunin

The existence of a human mRNA corresponding to the putative human  
25 bikunin nucleotide sequence inferred from the analysis of Figure 3, was confirmed as follows. The nucleic acid primer hybridizing 5' to the Kunitz-encoding cDNA sequence of R35464 (b.p. 3-27 of consensus nucleotide sequence in Figure 3): GGTCTAGAGGCCGGTCGTTCTGCCTGGCTGGGA (a 5' primer derived from R35464 sequence with an XbaI site; SEQ ID NO.: 35), and  
30 the nucleic acid primer hybridizing 3' to the Kunitz encoding sequence of R74593 (b.p. 680-700 of consensus nucleotide sequence in Figure 3), was used to PCR amplify, from a Clontech human placental library, a fragment of the size (ca. 670 b.p) expected from a cDNA consensus nucleotide sequence encoding the placental bikunin sequence of Figure 3 (Shown schematically in Figure 4A).

35 Using a 5' primer hybridizing to a sequence in R87894 that is 126 b.p 5' to the putative ATG start site discussed above, (shown schematically in Figure 4A at b.p. 110) plus the same 3' primer to R74593 as used above, it was possible to

amplify a fragment from a Clontech human placental library of the expected size (approximately 872 b.p) predicted by EST overlay (Shown schematically in Figure 4).

Sequencing of the 872 b.p. fragment showed it to contain nucleotide segment corresponding to b.p. 110 to 218 of EST R87894 at its 5' end and b.p. 310 to 542 of the consensus sequence for placental bikunin inferred from the EST overlay analysis (of Figure 3), at its 3' end. This 3' nucleotide sequence contained all of the Kunitz-like domain encoded by placental bikunin (102-159).

To obtain a cDNA encoding the entire extracellular region of the protein, the following 5' PCR primer: CACCTGATCGCGAGACCCC (SEQ ID NO.: 36) designed to hybridize to a sequence within EST R34808 was used with the same 3' primer to EST 74593 to amplify (30 cycles) an approximately 780 base-pair cDNA product from the human placental cDNA library. This product was gel purified, and cloned into the TA vector (Invitrogen) for DNA sequencing by the dideoxy method (Sanger F., et al., (1977) Proc. Natl. Acad. Sci (USA), 74, pp 5463-5467) with the following primers:

Vector Specific: GATTTAGGTGACACTATAG (SP6) (SEQ ID NO.: 37)  
TAATACGACTCACTATAGGG (T7) (SEQ ID NO.: 38)

Gene Specific: TTACCTGACCAAGGAGGAGTGC (SEQ ID NO.: 39)  
AATCCGCTGCATTCTGCTGGTG (SEQ ID NO.: 40)  
CAGTCACTGGGCCTGCCGT (SEQ ID NO.: 41)

The resulting cDNA sequence is depicted in Figure 4E together with its translation product. At the nucleotide level, the sequence exhibited only minor differences from the consensus EST sequence (Figure 4D). Translation of the sequence yielded a coding sequence containing an in-frame initiator ATG site, signal peptide and mature placental bikunin sequence and transmembrane domain. The translated sequence of the PCR product was missing the last 12 amino acid residues from the cytoplasmic domain as a consequence of the choice of selection of the 3' primer for PCR amplification. This choice of 3' PCR primer (designed based on the sequence of R74593) was also responsible for the introduction of an artificial S to F mutation at amino acid position 211 of the translated PCR-derived sequence. The signal peptide deduced from translation of the PCR fragment was somewhat different to that of the EST consensus.

To obtain a full length placental bikunin cDNA, the PCR derived product

(Figure 4E) was gel purified and used to isolate a non-PCR based full length clone representing the bikunin sequence. The PCR derived cDNA sequence was labeled with  $^{32}\text{P}$ -CTP by High Prime (Boehringer Mannheim) and used to probe a placental cDNA Library (Stratagene, Unizap<sup>TM</sup>  $\lambda$  library) using colony hybridization techniques. Approximately  $2 \times 10^6$  phage plaques underwent 3 rounds of screening and plaque purification. Two clones were deemed full length (~1.5 kilobases) as determined by restriction enzyme analysis and based on comparison with the size of the EST consensus sequence (see above). Sequencing of one of these clone by the dideoxy method yielded the oligonucleotide sequence depicted in Figure 4F. The translation product from this sequence yielded a protein with inframe initiator methionine, signal peptide and mature placental bikunin sequence. The mature placental bikunin sequence was identical to the sequence of the mature protein derived by translation of the EST consensus although the signal peptide sequence lengths and sequences differed. Unlike the PCR derived product, the cDNA derived by colony hybridization contained the entire ectodomain, transmembrane domain, cytoplasmic domain and in-frame stop codon. In fact, the clone extended all the way to the poly-A tail. The initiator methionine was followed by a hydrophobic signal peptide which was identical to the signal peptide encoded in the PCR derived clone. Subsequently we expressed and purified a soluble fragment of placental bikunin, bikunin (1-170), from Sf9 cells (Example 9), and found it to be a functional protease inhibitor (Example 10). Furthermore, we isolated from human placenta a soluble fragment of placental bikunin which was also an active protease inhibitor (Example 7).

Based on the above observations, it seems that full length placental bikunin has the capacity to exist as a transmembrane protein on the surface of cells as well as a soluble protein. Other transmembrane proteins that contain Kunitz domains are known to undergo proteolytic processing to yield mixtures of soluble and membrane associated forms. These include two forms of the Amyloid Precursor Protein termed APP751 (Esch F., et al., (1990) Science, 248, pp 1122-1124) and APP 770 (Wang R., et al., (1991), J. Biol Chem, 266, pp16960-16964).

Contact activation is a process which is activated by exposure of damaged vascular surfaces to components of the coagulation cascade. Angiogenesis is a process that involves local activation of plasmin at endothelial surfaces. The specificity of placental bikunin and its putative capacity to anchor to cell surfaces, suggest that the physiologic functions of transmembranous placental

bikunin may include regulation of contact activation and angiogenesis.

The amino acid sequences for placental bikunin (7-64), bikunin (102-159), and full length placental bikunin (Figure 4F) were searched against the PIR (Vers. 46.0) and PatchX (Vers. 46.0) protein databases as well as the GeneSeq (Vers. 20.0) protein database of patented sequences using the Genetics Computer Group program FastA. Using the Genetics Computer Group program TFastA (Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85:2444-2448), these same protein sequences were searched versus the six-frame translations of the GenBank (Vers. 92.0 with updates to 1/26/96) and EMBL (modified Vers. 45.0) nucleotide databases as well as the GeneSeq (Vers. 20.0) nucleotide database of patented sequences. The EST and STS subsets of GenBank and EMBL were not included in this set of searches. The best matches resulting from these searches contained sequences which were only about 50% identical over their full length to the 58-amino acid protein sequence derived from our analysis of clones R74593 and R35464.

### Isolation of Human Bikunin

As mentioned above, synthetic peptides corresponding to bikunin (7-64) and bikunin (102-159) as determined from the translated consensus sequence for bikunin (Figure 3), could be refolded (Examples 2 and 1, respectively) to yield active kallikrein inhibitor protein (Example 4 and 3, respectively). We exploited this unexpected property to devise a purification scheme to isolate native placental bikunin from human tissue.

Using a purification scheme which employed kallikrein-sepharose affinity chromatography as a first step, highly purified native potent kallikrein inhibitor was isolated. The isolated native human bikunin had an identical N-terminus (sequenced for 50 amino acid residues) as the sequence predicted by the translation of the consensus nucleic acid sequence (Figure 3) amino acid residues +1 to +50 (Example 7). This confirmed for the first time the existence of a novel native kallikrein inhibitor isolated from human placenta.

Known Kunitz-like domains are listed below. Residues believed to be making contact with target proteases are highlighted as of special interest (bold/underlined). These particular residues are named positions Xaa<sup>1-16</sup> for specific reference as shown by label Xaa.

#### Bikunin (7-64) (SEQ ID NO.: 4)

Xaa	1	2	3	4	5	6
	1	0	1	234	5	

IHDFCLVSKVY GRCRASMPRW WYNVTDGSCQ LFVYGGCDGN SNNYLTKEEC LKKCATV

Bikunin (102-159) (SEQ ID NO.: 6)

YEEYCTANAVT GPCRASFPRW YFDVERN SCN NFIYGGCRGN KNSYRSEEAC MLRCFRO

5

Tissue factor pathway inhibitor precursor 1 (SEQ ID NO.: 18)

-HSFCAFKADD GPKAIMKRF FFNIFTRQCE EFTIYGGCEGN QNRFESLEEC KKMCTRD

10 Tissue factor pathway inhibitor precursor 1 (SEQ ID NO.: 19)

-PDFCFLEEDP GICRGYITRY FYNNQTKQCE RFKYGGCLGN MNNFETLEEC KNICEDG

Tissue factor pathway inhibitor precursor (SEQ ID NO.: 20)

-PSWCLTPADR GLCRANENRF YYNSVIGKCR PFKYSGCGGN ENNFTSKQEC LRACKKG

15

Tissue factor pathway inhibitor precursor 2 (SEQ ID NO.: 21)

-AEICLLPLDY GPCRALLRY YYRYRTQSCR QFLYGGCEGN ANNFYTWEAC DDACWRI

Tissue factor pathway inhibitor precursor 2 (SEQ ID NO.: 22)

20

-PSFCYSPKDE GLCSANVTRY YFNPRYRTCD AFTYTGCGGN DNNFYSREDC KRACAKA

Amyloid precursor protein homologue (SEQ ID NO: 23)

-KAVCSQEAMT GPCRAVMPRT TFDLSKGKCV RFITGGCGGN RNNFESEDYC MAVCKAM

25

Aprotinin (SEQ ID NO: 24)

RPDFCLEPPYT GPKARIIRY FYNAKAGLCQ TFVYGGCRAK RNNFKSAEDC MRTCGGA

Inter-α-trypsin inhibitor precursor (SEQ ID NOs: 25)

-----CQLGYS A GPCMGMTSRY FYNGTSMACE TFQYGGCMGN GNNFYTEKEC LQTC

30

Inter-α-trypsin inhibitor precursor (SEQ ID NOs: 26)VAAACNLPIVR GPCRAFIOLW

AFDAVKGKCV LFPYGGCQGN GNKFYSEKEC REYCGVP

Amyloid precursor protein (SEQ ID NO: 27)

35

-EVCCSEQAET GPCRAMISRW YFDVTEGKCA PFFYGGCGGN RNNFDTEEYC MAVCGSA

Collagen α-3(VI) precursor (SEQ ID NO: 28)

----CKLPKDE GTCRDFILKW YYDPNTKSCA RFWYGGCGGN ENKFGSQKEC EKVC

HKI-B9 (SEQ ID NO: 29)

-PNVCAFPMEK GPCOTMTRW FFNFETGECE LFAYGGCGGN SNNFLRKEKC EKFCKFT

- 5 The placental bikunin, isolated domains or other variants of the present invention may be produced by standard solid phase peptide synthesis using either t-Boc chemistry as described by Merrifield R.B. and Barany G., in: The peptides, Analysis, Synthesis, Biology, 2, Gross E. et al., Eds. Academic Press (1980) Chapter 1; or using F-moc chemistry as described by Carpino L.A., and  
10 Han G.Y., (1970) J. Amer Chem Soc., 92, 5748-5749, and illustrated in Example 2. Alternatively, expression of a DNA encoding the placental bikunin variant may be used to produce recombinant placental bikunin variants.

The instant invention provides for the use of a purified human serine protease inhibitor which can specifically inhibit kallikrein, that has been isolated  
15 from human placental tissue via affinity chromatography. The human serine protein inhibitor, herein called human placental bikunin, contains two serine protease inhibitor domains of the Kunitz class. In one particular embodiment, the instant invention embodies a protein having the amino acid sequence:

20	ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN	50
	YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF	100
25	NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE	150
	ACMLRCFRQQ ENPPLPLGSK VVLAGAVS	179

(SEQ ID NO.: 1)

- 30 In a preferred embodiment the instant invention provides for native human placental bikunin protein having the amino acid sequence:

35	ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN	50
	YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF	100
40	NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE	150
	ACMLRCFRQQ ENPPLPLGSK	170

(SEQ ID NO.: 52)

In one aspect, the biological activity of the protein useful in practicing the instant invention is that it can bind to and substantially inhibit the biological

PCT/EP2003/002260

activity of trypsin, human plasma and tissue kallikreins, human plasmin and Factor XIIa. In a preferred embodiment, the present invention provides for a native human placental bikunin protein, in glycosylated form. In a further embodiment the instant invention encompasses native human bikunin protein  
5 which has been formed such that it contains at least one cysteine-cysteine disulfide bond. In a preferred embodiment, the protein contains at least one intra-chain cysteine-cysteine disulfide bond formed between a pair of cysteines selected from the group consisting of CYS11-CYS61, CYS20-CYS44, CYS36-CYS57, CYS106-CYS156, CYS115-CYS139, and CYS131-CYS152, wherein the  
10 cysteines are numbered according to the amino acid sequence of native human placental bikunin. One of ordinary skill will recognize that the protein of the instant invention may fold into the proper three-dimensional conformation, such that the biological activity of native human bikunin is maintained, where none, one or more, or all of the native intra-chain cysteine-cysteine disulfide bonds are  
15 present. In a most preferred embodiment, the protein of the instant invention is properly folded and is formed with all of the proper native cysteine-cysteine disulfide bonds.

Active protein for use in the instant invention can be obtained by purification from human tissue, such as placenta, or via synthetic protein chemistry techniques, as illustrated by the Examples below. It is also understood that the protein for use in the instant invention may be obtained using molecular biology techniques, where self-replicating vectors are capable of expressing the protein of the instant invention from transformed cells. Such protein can be made as non-secreted, or secreted forms from transformed cells. In order to facilitate secretion from transformed cells, to enhance the functional stability of the translated protein, or to aid folding of the bikunin protein, certain signal peptide sequences may be added to the NH<sub>2</sub>-terminal portion of the native human bikunin protein.  
20  
25

In one embodiment, the instant invention thus provides for the native  
30 human bikunin protein with at least a portion of the native signal peptide sequence intact. Thus one embodiment of the invention provides for native human bikunin with at least part of the signal peptide, having the amino acid sequence:

35	A G S F L   A W L G S   L L L S G   V L A	-1
	A D R E R   S I H D F   C L V S K   V V G R C   R A S M P	25
	R W W Y N   V T D G S   C Q L F V   Y G G C D   G N S N N	50
	Y L T K E   E C L K K   C A T V T   E N A T G   D L A T S	75
	R N A A D   S S V P S   A P R R Q   D S E D H   S S D M F	100
40	N Y E E Y   C T A N A   V T G P C   R A S F P   R W Y F D	125

V E R N S	C N N F I	Y G G C R	G N K N S	Y R S E E	150
A C M L R	C F R Q Q	E N P P L	P L G S K	V V V L A	175
G A V S					179

5 (SEQ ID NO.: 2)

In a preferred embodiment, the instant invention provides for the use of a native human placental bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO.: 52 with an intact leader segment having  
10 the amino acid sequence:

MAQLCGL RRSRAFLALL GSLLLGVLA -1

(SEQ ID NO.: 53)

15 In another embodiment, the instant invention provides for the use of bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO.: 52 with the intact leader segment having the amino acid sequence:

MLR AEADGVSRL GSLLLGVLA -1

20 (SEQ ID NO.: 54)

In a preferred numbering system used herein the amino acid numbered +1 is assigned to the NH<sub>2</sub>-terminus of the amino acid sequence for native human placental bikunin. One will readily recognize that functional protein fragments  
25 can be derived from native human placental bikunin, which will maintain at least part of the biological activity of native human placental bikunin, and act as serine protease inhibitors.

In one embodiment, the protein for use in the method of the instant invention comprises a fragment of native human placental bikunin, which  
30 contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 7-159, hereinafter called "bikunin (7-159)". Thus the instant invention embodies a method that employs a protein having the amino acid sequence:

I H D F	C L V S K	V V G R C	R A S M P	25	
R W W Y N	V T D G S	C Q L F V	Y G G C D	G N S N N	50
Y L T K E	E C L K K	C A T V T	E N A T G	D L A T S	75
R N A A D	S S V P S	A P R R Q	D S E D H	S S D M F	100
N Y E E Y	C T A N A	V T G P C	R A S F P	R W Y F D	125
40 V E R N S	C N N F I	Y G G C R	G N K N S	Y R S E E	150
A C M L R	C F R Q				159

(SEQ ID NO.: 3)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another functional variant of this embodiment can be the fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 11-156, bikunin (11-156)

15                  *H. S. H. B. R. C.*

One can recognize that the individual Kunitz-like domains are also fragments of the native placental bikunin. In particular, the instant invention contemplates the use of a protein having the amino acid sequence of a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 7-64, hereinafter called "bikunin (7-64)". Thus in one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

30 (SEO ID NO.; 4)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of the protein of the instant invention can be a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 11-61, "bikunin (11-61)" having the amino acid sequence:

40	C L V S K    V V G R C    R A S M P R W W Y N    V T D G S    C Q L F V    Y G G C D    G N S N N Y L T K E    E C L K K    C	25 50 61
----	---	----------------

(SEQ ID NO.: 5)

The instant invention also provides for a protein having the amino acid sequence of a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 102-159, hereinafter called "bikunin (102-159)". Thus one embodiment the instant invention encompasses a protein  
5 which contains at least one Kunitz-like domain having the amino acid sequence:

10           Y E E Y C T A N A V T G P C R A S F P R W Y F D         125  
         V E R N S C N N F I Y G G C R G N K N S Y R S E E         150  
         A C M L R C F R Q   159

(SEQ ID NO.: 6)

where the amino acid numbering corresponds to that of the amino acid  
15 sequence of native human placental bikunin. Another form of this domain can  
be a Kunitz-like domain consisting of the amino acid sequence of native human  
placental bikunin amino acids 106-156, "bikunin (106-156)" having the amino  
acid sequence:

20           C T A N A V T G P C R A S F P R W Y F D         125  
         V E R N S C N N F I Y G G C R G N K N S Y R S E E         150  
         A C M L R C   156

(SEQ ID NO.: 7)

25           Thus one of ordinary skill will recognize that fragments of the native  
         human bikunin protein can be made which will retain at least some of the native  
         protein biological activity. Such fragments can also be combined in different  
         orientations or multiple combinations to provide for alternative proteins which  
30           retain some of, the same, or more biological activity of the native human bikunin  
         protein.

35           One will readily recognize that biologically active protein employed in  
         the method of the instant invention may comprise one or more of the instant  
         Kunitz-like domains in combination with additional Kunitz-like domains from  
         other sources. Biologically active protein of the method of the instant invention  
         may comprise one or more of the instant Kunitz-like domains in combination  
         with additional protein domains from other sources with a variety of biological  
         activities. The biological activity of the protein useful in practicing the instant  
         invention can be combined with that of other known protein or proteins to  
40           provide for multifunctional fusion proteins having predictable biological

activity. Thus, in one embodiment, the method of instant invention encompasses the use of a protein which contains at least one amino acid sequence segment the same as, or functionally equivalent to the amino acid sequence of either SEQ ID NO.: 5 or SEQ ID NO.: 7.

5 An open reading frame which terminates at an early stop codon can still code for a functional protein. The instant invention encompasses such alternative termination, and in one embodiment provides for the use of a protein of the amino acid sequence:

10 A D R E R S I H D F C L V S K V V G R C R A S M P 25  
R W W Y N V T D G S C Q L F V Y G G C D G N S N N 50  
Y L T K E E C L K K C A T V T E N A T G D L A T S 75  
R N A A D S S V P S A P R R Q D S 92

15 (SEQ ID NO.: 8)

In one embodiment, the instant invention provides for the use of substantially purified, or recombinantly produced native human bikunin protein with an intact segment of the leader sequence, and at least a portion of the native 20 transmembrane region intact. Thus one embodiment of the invention provides for the use of native human bikunin, with an intact leader sequence, and with at least part of the transmembrane domain (underlined), having an amino acid sequence selected from:

25 EST MLR AEADGVSRLL GSLLLGVLA -1  
PCR MAQLCGL RRSRAFLALL GSLLLGVLA -1  
λcDNA MAQLCGL RRSRAFLALL GSLLLGVLA -1  
  
EST ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
PCR ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
30 λcDNA ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
  
EST YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100  
PCR YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100  
35 λcDNA YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100  
  
EST NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
PCR NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
λcDNA NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
  
40 EST ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200  
PCR ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200  
λcDNA ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200  
  
45 EST QERALRTVWS SGDDKEQLVK NTYVL 225  
PCR QERALRTVWS FGD 213  
λcDNA QERALRTVWS SGDDKEQLVK NTYVL 225

where EST is EST derived consensus SEQ ID NO.: 45, PCR is PCR clone SEQ ID

NO.:47, and λcDNA is lambda cDNA clone SEQ ID NO.:49. In a preferred embodiment a protein of the method of the instant invention comprises one of the amino acid sequence of SEQ ID NO.: 45, 47 or 49 wherein the protein has been cleaved in the region between the end of the last Kunitz domain and the transmembrane region (underlined).

The instant invention also embodies the use of the protein wherein the signal peptide is deleted. Thus the method of the instant invention provides for a protein having the amino acid sequence of SEQ ID NO.: 52 continuous with a transmembrane amino acid sequence:

10 EST           VVVLAGLFVM VLILFLGASM VYLIRVARRN           200  
EST           QERALRTVWS SGDDKEQLVK NTYVL           225

(SEQ ID NO.: 69)

15 a transmembrane amino acid sequence:

20 PCR           VVVLAGLFVM VLILFLGASM VYLIRVARRN           200  
PCR           QERALRTVWS FGD           213

(SEQ ID NO.: 68)

or a transmembrane amino acid sequence:

25 λcDNA           VVVLAGLFVM VLILFLGASM VYLIRVARRN           200  
λcDNA           QERALRTVWS SGDDKEQLVK NTYVL           225

(SEQ ID NO.: 67).

30 The protein amino acid sequences for use in the instant invention clearly teach one skilled in the art the appropriate nucleic acid sequences which can be used in molecular biology techniques to produce the proteins for use in the instant invention. Thus, one embodiment of the instant invention provides for use of a nucleic acid sequence which encodes for a human bikunin having the consensus DNA sequence of Figure 3 (SEQ ID NO.: 9), which translates into the amino acid sequence for native human placental bikunin sequence of Figure 3 (SEQ ID NO.: 10). In another embodiment, the instant invention provides for a consensus nucleic acid sequence of Figure 4C (SEQ ID NO.: 51) which encodes for an amino acid sequence of Figure 4D (SEQ ID NO.: 45).

35 40 In a preferred embodiment, the instant invention provides for the use of a nucleic acid sequence which encodes for native human placental bikunin having the DNA sequence of Figure 4F (SEQ ID NO.: 48) which encodes for the protein sequence of SEQ ID NO.: 49. In an another embodiment, the instant invention provides for a nucleic acid sequence of Figure 4E (SEQ ID NO.: 46) which

encodes for a protein sequence of SEQ ID NO.: 47.

One can easily recognize that certain allelic mutations, and conservative substitutions made in the nucleic acid sequence can be made which will still result in a protein amino acid sequence encompassed by the method of the instant invention. One of skill in the art can recognize that certain natural allelic mutations of the protein of the instant invention, and conservative substitutions of amino acids in the protein of the instant invention will not significantly alter the biological activity of the protein, and are encompassed by the instant invention.

The instant invention also provides for pharmaceutical compositions containing human placental bikunin and fragments thereof that are useful for stimulating MCC in patients impaired by mucociliary dysfunction.

The present invention also provides methods for stimulating MCC in a patient suffering from mucociliary dysfunction, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention in a biologically compatible vehicle is administered to the patient.

The present invention also provides for a method for stimulating MCC that employs variants of placental bikunin, and the specific Kunitz domains described above, that contain amino acid substitutions that alter the protease specificity. Preferred sites of substitution are indicated below as positions Xaa<sup>1</sup> through Xaa<sup>32</sup> in the amino acid sequence for native placental bikunin. Substitutions at Xaa<sup>1</sup> through Xaa<sup>16</sup> are also preferred for variants of bikunin (7-64), while substitutions at Xaa<sup>17</sup> through Xaa<sup>32</sup> are preferred for variants of bikunin (102-159).

Thus the method of the present invention embodies the use of a protein having an amino acid sequence:

Ala Asp Arg Glu Arg Ser Ile Xaa <sup>1</sup> Asp Phe	10
Cys Leu Val Ser Lys Val Xaa <sup>2</sup> Gly Xaa <sup>3</sup> Cys	20
Xaa <sup>4</sup> Xaa <sup>5</sup> Xaa <sup>6</sup> Xaa <sup>7</sup> Xaa <sup>8</sup> Xaa <sup>9</sup> Trp Trp Tyr Asn	30
Val Thr Asp Gly Ser Cys Gln Leu Phe Xaa <sup>10</sup>	40
Tyr Xaa <sup>11</sup> Gly Cys Xaa <sup>12</sup> Xaa <sup>13</sup> Xaa <sup>14</sup> Ser Asn Asn	50
Tyr Xaa <sup>15</sup> Thr Lys Glu Glu Cys Leu Lys Lys	60
Cys Ala Thr Xaa <sup>16</sup> Thr Glu Asn Ala Thr Gly	70
Asp Leu Ser Thr Ser Arg Asn Ala Ala Asp	80
Ser Ser Val Pro Ser Ala Pro Arg Arg Gln	90
Asp Ser Glu His Asp Ser Ser Asp Met Phe	100
Asn Tyr Xaa <sup>17</sup> Glu Tyr Cys Thr Ala Asn Ala	110

	Val Xaa <sup>18</sup> Gly Xaa <sup>19</sup> Cys Xaa <sup>20</sup> Xaa <sup>21</sup> Xaa <sup>22</sup> Xaa <sup>23</sup> Xaa <sup>24</sup>	120
	Xaa <sup>25</sup> Trp Tyr Phe Asp Val Glu Arg Asn Ser	130
	Cys Asn Asn Phe Xaa <sup>26</sup> Tyr Xaa <sup>27</sup> Gly Cys Xaa <sup>28</sup>	140
	Xaa <sup>29</sup> Xaa <sup>30</sup> Lys Asn Ser Tyr Xaa <sup>31</sup> Ser Glu Glu	150
5	Ala Cys Met Leu Arg Cys Phe Arg Xaa <sup>32</sup> Gln	160
	Glu Asn Pro Pro Leu Pro Leu Gly Ser Lys	170
	Val Val Val Leu Ala Gly Ala Val Ser	179
	(SEQ ID NO: 11).	

- 10 where Xaa<sup>1</sup> - Xaa<sup>32</sup> each independently represents a naturally occurring amino acid residue except Cys, with the proviso that at least one of the amino acid residues Xaa<sup>1</sup>-Xaa<sup>32</sup> is different from the corresponding amino acid residue of the native sequence.

In the present context, the term "naturally occurring amino acid residue" is intended to indicate any one of the 20 commonly occurring amino acids, i.e., Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

By substituting one or more amino acids in one or more of the positions indicated above, it may be possible to change the inhibitor specificity profile of native placental bikunin or that of the individual Kunitz-like domains, bikunin(7-64) or bikunin (102-159) so that it preferentially inhibits other serine proteases such as, but not limited to, the enzymes of the complement cascade, TF/FVIIa, FXa, prostasin, thrombin, neutrophil elastase, cathepsin G or proteinase-3.

Examples of preferred variants of placental bikunin include those wherein Xaa<sup>1</sup> is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Val or Lys, in particular wherein Xaa<sup>1</sup> is His or Pro; or wherein Xaa<sup>2</sup> is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala, Lys, in particular wherein Xaa<sup>2</sup> is Val or Thr; or wherein Xaa<sup>3</sup> is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu, Thr, in particular wherein Xaa<sup>3</sup> is Arg or Pro; or wherein Xaa<sup>4</sup> is an amino acid residue selected from the group consisting of Arg, Lys and Ser, Gln, in particular wherein Xaa<sup>4</sup> is Arg or Lys; or wherein Xaa<sup>5</sup> is an amino acid residue selected from the group consisting of Ala, Gly, Asp, Thr, in particular wherein Xaa<sup>5</sup> is Ala; or wherein Xaa<sup>6</sup> is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg, Phe, in particular wherein Xaa<sup>6</sup> is Ser or Arg ; or wherein Xaa<sup>7</sup> is an amino acid residue selected from the group

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consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa<sup>7</sup> is Met or Ile; or wherein Xaa<sup>8</sup> is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Gln, Asn, Leu, Ser or Ile, in particular wherein Xaa<sup>8</sup> is Pro or Ile; or wherein Xaa<sup>9</sup> is an amino acid residue selected from the group  
5 consisting of Arg, Lys or Leu, in particular wherein Xaa<sup>9</sup> is Arg; or wherein Xaa<sup>10</sup> is an amino acid residue selected from the group consisting of Val, Ile, Lys, Ala, Pro, Phe, Trp, Gln, Leu and Thr, in particular wherein Xaa<sup>10</sup> is Val; or wherein Xaa<sup>11</sup> is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa<sup>11</sup> is Gly; or wherein Xaa<sup>12</sup> is an  
10 amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gln, Gly, in particular wherein Xaa<sup>12</sup> is Asp or Arg; or wherein Xaa<sup>13</sup> is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa<sup>14</sup> is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa<sup>15</sup> is an amino acid residue selected from the group  
15 consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa<sup>15</sup> is Leu or Lys; or wherein Xaa<sup>16</sup> is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Val, in particular wherein Xaa<sup>16</sup> is Val or Ala; or wherein Xaa<sup>17</sup> is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Lys and Val, in  
20 particular wherein Xaa<sup>17</sup> is Glu or Pro; or wherein Xaa<sup>18</sup> is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala or Lys, in particular wherein Xaa<sup>18</sup> is Thr; or wherein Xaa<sup>19</sup> is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu or Thr, in particular wherein Xaa<sup>19</sup> is Pro; or wherein Xaa<sup>20</sup> is an amino acid residue  
25 selected from the group consisting of Arg, Lys, Gln and Ser, in particular wherein Xaa<sup>20</sup> is Arg or Lys; or wherein Xaa<sup>21</sup> is an amino acid residue selected from the group consisting of Ala, Asp, Thr or Gly ; in particular wherein Xaa<sup>21</sup> is Ala; or wherein Xaa<sup>22</sup> is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg or Phe, in particular wherein Xaa<sup>22</sup>  
30 is Ser or Arg ; or wherein Xaa<sup>23</sup> is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa<sup>23</sup> is Phe or Ile; or wherein Xaa<sup>24</sup> is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Asn, Leu, Gln, Ser or Ile, in particular wherein Xaa<sup>24</sup> is Pro or Ile; or wherein Xaa<sup>25</sup> is an amino acid residue selected from the group  
35 consisting of Arg, Lys or Leu, in particular wherein Xaa<sup>25</sup> is Arg; or wherein Xaa<sup>26</sup> is an amino acid residue selected from the group consisting of Val, Ile, Lys, Leu, Ala, Pro, Phe, Gln, Trp and Thr, in particular wherein Xaa<sup>26</sup> is Val or

Ile; or wherein Xaa<sup>27</sup> is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa<sup>27</sup> is Gly; or wherein Xaa<sup>28</sup> is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gly or Gln, in particular wherein Xaa<sup>28</sup> is Arg; or wherein Xaa<sup>29</sup> is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa<sup>30</sup> is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa<sup>31</sup> is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa<sup>31</sup> is Arg or Lys; or wherein Xaa<sup>32</sup> is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Thr, in particular wherein Xaa<sup>32</sup> is Gln or Ala.

The invention also relates to DNA constructs that encode the Placental bikunin protein variants of the present invention. These constructs may be prepared by synthetic methods such as those described in Beaucage S.L. and Caruthers M.H., (1981) Tetrahedron Lett, 22, pp1859-1862; Matteucci M.D and Caruthers M.H., (1981), J. Am. Chem. Soc. 103, p 3185; or from genomic or cDNA which may have been obtained by screening genomic or cDNA libraries with cDNA probes designed to hybridize with placental bikunin encoding DNA sequence. Genomic or cDNA sequence can be modified at one or more sites to obtain cDNA encoding any of the amino acid substitutions or deletions described in this disclosure.

The instant invention also relates to expression vectors containing the DNA constructs encoding the placental bikunin, isolated domains or other variants of the present invention that can be used for the production of recombinant placental bikunin variants. The cDNA should be connected to a suitable promoter sequence which shows transcriptional activity in the host cell of choice, possess a suitable terminator and a poly-adenylation signal. The cDNA encoding the placental bikunin variant can be fused to a 5' signal peptide that will result in the protein encoded by the cDNA to undergo secretion. The signal peptide can be one that is recognized by the host organism. In the case of a mammalian host cell, the signal peptide can also be the natural signal peptide present in full length placental bikunin. The procedures used to prepare such vectors for expression of placental bikunin variants are well known in the art and are for example described in Sambrook et al., Molecular Cloning: A laboratory Manual, Cold Spring Harbor, New York, (1989).

The instant invention also relates to transformed cells containing the DNA constructs encoding the placental bikunin, isolated domains or other

variants of the present invention that can be used for the production of recombinant placental bikunin variants. A variety of combinations of expression vector and host organism exist which can be used for the production of the placental bikunin variants. Suitable host cells include baculovirus infected Sf9 5 insect cells, mammalian cells such as BHK, CHO, Hela and C-127, bacteria such as E. coli, and yeasts such as Saccharomyces cervisiae. Methods for the use of mammalian, insect and microbial expressions systems needed to achieve expression of placental bikunin are well known in the art and are described, for example, in Ausubel F.M et al., Current Protocols in Molecular Biology, John 10 Wiley & Sons (1995), Chapter 16. For fragments of placental bikunin containing a single Kunitz inhibitor domain such as bikunin (7-64) and (102-159), yeast and E. coli expression systems are preferable, with yeast systems being most preferred. Typically, yeast expression would be carried out as described in US 15 patent 5,164,482 for aprotinin variants and adapted in Example 5 of the present specification for placental bikunin (102-159). E.coli expression could be carried out using the methods described in US patent 5,032,573. Use of mammalian and yeast systems are most preferred for the expression of larger placental bikunin variants containing both inhibitor domains such as the variant bikunin(7-159).

DNA encoding variants of placental bikunin that possess amino acid 20 substitution of the natural amino sequence can be prepared for expression of recombinant protein using the methods of Kunkel T.A., (1985) Proc. Natl. Acad. Sci USA 82, pp 488-492. Briefly, the DNA to be mutagenized is cloned into a single stranded bacteriophage vector such as M13. An oligonucleotide spanning the region to be changed and encoding the substitution is hybridized to the 25 single stranded DNA and made double stranded by standard molecular biology techniques. This DNA is then transformed into an appropriate bacterial host and verified by dideoxynucleotide sequencing. The correct DNA is then cloned into the expression plasmid. Alternatively, the target DNA may be mutagenized by standard PCR techniques, sequenced, and inserted into the appropriate 30 expression plasmid.

The following particular examples are offered by way of illustration, and not limitation, of certain aspects and preferred embodiments of the instant invention.

35 **Example 1**  
**Preparation of synthetic placental bikunin (102-159)**  
*Materials and methods/Reagents used.* The fluorogenic substrate Tos-

Gly-Pro-Lys-AMC was purchased from Bachem BioScience Inc (King of Prussia, PA). PNGB, Pro-Phe-Arg-AMC, Ala-Ala-Pro-Met-AMC, bovine trypsin (type III), human plasma kallikrein, and human plasmin were from Sigma (St. Louis, MO).

- 5 Recombinant aprotinin (Trasylol®) was from Bayer AG (Wuppertal, Germany). Pre-loaded Gln Wang resin was from Novabiochem (La Jolla, CA). Thioanisole, ethanedithiol and t-butyl methyl ether was from Aldrich (Milwaukee, WI).
- 10 *Quantification of functional placental bikunin (7-64) and (102-159)*  
The amount of trypsin inhibitory activity present in the refolded sample at various stages of purification was measured using GPK-AMC as a substrate. Bovine trypsin (200 pmoles) was incubated for 5 min at 37°C with bikunin (7-64) or (102-159), from various stages of purification, in buffer A (50 mM Hepes, pH 7.5, 0.1 M NaCl, 2 mM CaCl<sub>2</sub> and 0.01% triton X-100). GPK-AMC was added (20 µM final) and the amount of coumarin produced was determined by measuring the fluorescence (ex = 370 nm, em = 432 nm) on a Perkin-Elmer LS-50B fluorimeter over a 2 min. period. For samples being tested the % inhibition for each was calculated according to equation 1; where R<sub>O</sub> is the rate of  
15 fluorescence increase in the presence of inhibitor and R<sub>I</sub> is the rate determined in the absence of added sample. One unit of activity for the inhibitor is defined as the amount needed to achieve 50% inhibition in the assay using the conditions as described.
- 20  
25 % inhibition = 100 × [1 - R<sub>O</sub>/R<sub>I</sub>] (1)

*Synthesis.* Placental bikunin (102-159) was synthesized on an Applied Biosystems model 420A peptide synthesizer using NMP-HBTU Fmoc chemistry. The peptide was synthesized on pre loaded Gln resin with an 8-fold excess of  
30 amino acid for each coupling. Cleavage and deprotection was performed in 84.6% trifluoroacetic acid (TFA), 4.4% thioanisole, 2.2% ethanedithiol, 4.4% liquified phenol, and 4.4% H<sub>2</sub>O for 2 hours at room temperature. The crude peptide was precipitated, centrifuged and washed twice in t-butyl methyl ether. The peptide was purified on a Dynamax 60A C18 reverse-phase HPLC column  
35 using a TFA/acetonitrile gradient. The final preparation (61.0 mg) yielded the correct amino acid composition and molecular mass by Electrospray mass spectroscopy (MH<sup>+</sup> = 6836.1; calcd = 6835.5) for the predicted sequence:

YEEYCTANAV TGPCRASFPR WYFDVERNSC NNFIYGGCRG NKNSYRSEEA  
CMLRCFRQ (SEQ ID NO.: 6)

5        **Purification.** Refolding of placental bikunin (102-159) was performed according to the method of Tam et al., (J. Am. Chem. Soc. 1991, 113; 6657-62). A portion of the purified peptide (15.2 mg) was dissolved in 4.0 ml of 0.1 M Tris, pH 6.0, and 8 M urea. Oxidation of the disulfides was accomplished by dropwise addition of a solution containing 23% DMSO, and 0.1 M Tris, pH 6.0

10      to obtain a final concentration of 0.5 mg/ml peptide in 20% DMSO, 0.1 M Tris, pH 6.0, and 1 M urea. The solution was allowed to stir for 24 hr at 25°C after which it was diluted 1:10 in buffer containing 50 mM Tris, pH 8.0, and 0.1 M NaCl. The material was purified using a kallikrein affinity column made by covalently attaching 30 mg of bovine pancreatic kallikrein (Bayer AG) to 3.5 mls

15      of CNBr activated Sepharose (Pharmacia) according to the manufacturers instructions. The refolded material was loaded onto the affinity column at a flow rate of 1 ml/min and washed with 50 mM Tris, pH 8.0, and 0.1 M NaCl until absorbance at 280 nm of the wash could no longer be detected. The column was eluted with 3 volumes each of 0.2 M acetic acid, pH 4.0 and 1.7. Active

20      fractions were pooled (see below) and the pH of the solution adjusted to 2.5. The material was directly applied to a Vydac C18 reverse-phase column (5 micron, 0.46 x 25 cm) which had been equilibrated in 22.5% acetonitrile in 0.1% TFA. Separation was achieved using a linear gradient of 22.5 to 40% acetonitrile in 0.1% TFA at 1.0 ml/min over 40 min. Active fractions were pooled, lyophilized,

25      redissolved in 0.1% TFA, and stored at -20°C until needed.

Results.     Synthetic placental bikunin (102-159) was refolded using 20% DMSO as the oxidizing agent as described above, and purified by a 2-step purification protocol as shown below, to yield an active trypsin inhibitor (Table 1 below).

**Table 1 Purification table for the isolation of synthetic placental bikunin (102-159)**

TABLE 1						
Purification Step	Vol (ml)	mg/ml	mg	Units <sup>c</sup> (U)	SpA (U/mg)	Yield
8.0 M Urea	4.0	3.75 <sup>a</sup>	15.0	0	0	-
20% DMSO	32.0	0.47 <sup>a</sup>	15.0	16,162	1,078	100
Kallikrein affinity	9.8	0.009 <sup>b</sup>	0.09	15,700	170,000	97
C18	3.0	0.013 <sup>ab</sup>	0.04	11,964	300,000	74

<sup>a</sup>Protein determined by AAA.

5       <sup>b</sup>Protein determined by OD280 nm using the extinction coefficient determined for the purified protein ( $1.7 \times 10^4 \text{ Lmol}^{-1} \text{ cm}^{-1}$ ).

<sup>c</sup>One Unit is defined as the amount of material required to inhibit 50% of trypsin activity in a standard assay.

10           Chromatography of the crude refolded material over an immobilized bovine pancreatic kallikrein column selectively isolated 6.0% of the protein and 97% of the trypsin inhibitory activity present. Subsequent chromatography using C18 reverse-phase yielded a further purification of 2-fold, with an overall recovery of 74%. On RPHPLC, the reduced and refolded placental bikunin (102-159), exhibited elution times of 26.3 and 20.1 minutes, respectively. Mass spectroscopy analysis of the purified material revealed a molecular mass of 6829.8; a loss of 6 mass units from the starting material. This demonstrates the complete formation of the 3 disulfides predicted from the peptide sequence.

15           The isoelectric points of the purified, refolded synthetic placental bikunin (102-159) was determined using a Multiphor II Electrophoresis System (Pharmacia) run according to the manufacturers suggestions, together with pI standards, using a precast Ampholine® PAGplate (pH 3.5 to 9.5) and focused for 1.5 hrs. After staining, the migration distance from the cathodic edge of the gel to the different protein bands was measured. The pI of each unknown was 20 determined by using a standard curve generated by a plot of the migration distance of standards versus the corresponding pI's. With this technique, the pI of placental bikunin (102-159) was determined to be 8.3, in agreement with the value predicted from the amino acid sequence. This is lower than the value of 10.5 established for the pI of aprotinin. (Tenstad et al., 1994, Acta Physiol. Scand. 25 152:33-50).

Example 2

**Preparation of synthetic placental bikunin (7-64)**

Placental bikunin (7-64) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) but with the following modifications: during refolding, the synthetic peptide was stirred for 30 hr as a solution in 20% DMSO at 25°C; purification by C18 RP-HPLC was achieved with a linear gradient of 25 to 45% acetonitrile in 0.1% TFA over 40 min (1ml/min). Active fractions from the first C18 run were reapplied to the column and fractionated with a linear gradient (60 min, 1 ml/min) of 20 to 40% acetonitrile in 0.1% TFA.

**Results.** The final purified reduced peptide exhibited an MH<sup>+</sup> = 6563, consistent with the sequence:

IHDFLVSKV VGRCRASMPR WWYNVTDGSC QLFVYGGCDG NSNNYLTKEE  
CLKKCATV (SEQ ID NO.: 4)

The refolding and purification yielded a functional Kunitz domain that was active as an inhibitor of trypsin (Table 2 below).

**Table 2A Purification table for the isolation of synthetic placental bikunin (7-64)**

TABLE 2A						
Purification Step	Vol (ml)	mg/ml	mg	Units (U)	SpA (U/mg)	Yield
8.0 M Urea	8.0	2.5	20.0	0	0	-
20% DMSO	64.0	0.31	20.0	68,699	3,435	100
Kall affinity pH 4.0	11.7	0.10	1.16	43,333	36,110	62
Kall affinity pH 1.7	9.0	0.64	5.8	4972	857	7.2
C18-1	4.6	0.14	0.06	21,905	350,143	31.9
C18-2	1.0	0.08	0.02	7,937	466,882	11.5

The purified refolded protein exhibited an MH<sup>+</sup> = 6558, i.e. 5±1 mass units less than for the reduced peptide. This demonstrates that refolding caused the formation of at least one appropriate disulfide bond.

The pI of placental bikunin (7-64) was determined using the methods employed to determine the pI of placental bikunin (102-159). Placental bikunin (7-64) exhibited a pI that was much higher than the predicted value (pI = 7.9). Refolded placental bikunin (7-64) migrated to the cathodic edge of the gel (pH

9.5) and an accurate pI could not be determined under these conditions.

#### *Continued Preparation of synthetic placental bikunin (7-64)*

Because the synthetic placental bikunin (7-64) may not have undergone complete deprotection prior to purification and refolding, refolding was repeated using protein which was certain to be completely deprotected. Placental bikunin (7-64) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) but with the following modifications: during refolding, the synthetic peptide (0.27 mg/ml) was stirred for 30 hr as a solution in 20% DMSO at 25°C; purification by C18 RP-HPLC was achieved with a linear gradient of 22.5 to 50% acetonitrile in 0.1% TFA over 40 min (1 ml/min).

**Results.** The final purified reduced peptide exhibited an  $MH^+ = 6567.5$ , consistent with the sequence:

IHDFLVSKV VGRCRASMPRW WYNVTDGSC QLFVYGGCDG NSNNYLTKEE  
CLKKCATV (SEQ ID NO.: 4)

The refolding and purification yielded a functional Kunitz domain that was as active as an inhibitor of trypsin (Table 2B below).

**Table 2B Purification table for the isolation of synthetic placental bikunin (7-64)**

TABLE 2B						
Purification Step	Vol (ml)	mg/ml	mg	Units (U)	SpA (U/mg)	Yield
8.0 M Urea	4.9	2.1	10.5	0	0	-
20% DMSO	39.0	0.27	10.5	236,000	22,500	100
Kallikrein Affinity (pH 2)	14.5	0.3	0.43	120,000	279,070	50.9
C18 Reverse-Phase	0.2	1.2	0.24	70,676	294,483	30.0

The purified refolded protein exhibited an  $MH^+ = 6561.2$ , i.e. 6.3 mass units less than for the reduced peptide. This demonstrates that refolding caused the formation of the expected three disulfide bonds.

The pI of refolded placental bikunin (7-64) was determined using the methods employed to determine the pI of placental bikunin (102-159). Refolded placental bikunin (7-64) exhibited a pI of 8.85, slightly higher than the predicted value (pI = 7.9).

### Example 3

#### In vitro specificity of functional placental bikunin fragment (102-159)

*Proteases.* Bovine trypsin, human plasmin, and bovine pancreatic kallikrein quantitation was carried out by active site titration using p-nitrophenyl p'-guanidinobenzoate HCl as previously described (Chase, T., and Shaw, E., (1970) *Methods Enzmol.* **19**, 20-27). Human kallikrein was quantitated by active site titration using bovine aprotinin as a standard and PFR-AMC as a substrate assuming a 1:1 complex formation. The  $K_m$  for GPK-AMC with trypsin and plasmin under the conditions used for each enzyme was 29  $\mu\text{M}$  and 726  $\mu\text{M}$ , respectively; the  $K_m$  for PFR-AMC with human plasma kallikrein and bovine pancreatic kallikrein was 457  $\mu\text{M}$  and 81.5  $\mu\text{M}$ , respectively; the  $K_m$  for AAPR-AMC with elastase was 1600  $\mu\text{M}$ . Human tissue kallikrein (Bayer, Germany) quantification was carried out by active site titration using p'nitrophenyl p'-guanidinobenzoate HCl as previously described (Chase, T., and Shaw, E., (1970) *Methods Enzmol.* **19**, 20-27).

*Inhibition Kinetics:* The inhibition of trypsin by placental bikunin (102-159) or aprotinin was measured by the incubation of 50 pM trypsin with placental bikunin (102-159) (0-2 nM) or aprotinin (0-3 nM) in buffer A in a total volume of 1.0 ml. After 5 min. at 37°C, 15  $\mu\text{l}$  of 2 mM GPK-AMC was added and the change in fluorescence (as above) was monitored. The inhibition of human plasmin by placental bikunin (102-159) and aprotinin was determined with plasmin (50 pM) and placental bikunin (102-159) (0-10 nM) or aprotinin (0-4 nM) in buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 0.02% triton x-100. After 5 min. incubation at 37°C, 25  $\mu\text{l}$  of 20 mM GPK-AMC was added and the change in fluorescence monitored. The inhibition of human plasma kallikrein by placental bikunin (102-159) or aprotinin was determined using kallikrein (2.5 nM) and placental bikunin (102-159) (0-3 nM) or aprotinin (0-45 nM) in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.02% triton x-100. After 5 min. at 37°C 15  $\mu\text{l}$  of 20 mM PFR-AMC was added and the change in fluorescence monitored. The inhibition of bovine pancreatic kallikrein by placental bikunin (102-159) and aprotinin was determined in a similar manner with kallikrein (92 pM), placental bikunin (102-159) (0-1.6 nM) and aprotinin (0-14 pM) and a final substrate concentration of 100  $\mu\text{M}$ . The apparent inhibition constant  $K_i^*$  was determined using the nonlinear regression data analysis program Enzfitter software (Biosoft, Cambridge, UK): The kinetic data from each experiment were analyzed in terms of the equation for a tight binding inhibitor:

$$V_i/V_o = 1 - (E_o + I_o + K_i^* - [(E_o + I_o + K_i^*)^2 - 4 E_o I_o]^{1/2})/2E_o \quad (2)$$

where  $V_i/V_o$  is the fractional enzyme activity (inhibited vs. uninhibited rate),

5 and  $E_o$  and  $I_o$  are the total concentrations of enzyme and inhibitor, respectively.  
Ki values were obtained by correcting for the effect of substrate according to the  
equation:

$$K_i = K_i^*/(1 + [S_o]/K_m) \quad (3)$$

10

(Boudier, C., and Bieth, J. G., (1989) *Biochim Biophys Acta.* 995: 36-41)

For the inhibition of human neutrophil elastase by placental bikunin (102-159) and aprotinin, elastase (19 nM) was incubated with placental bikunin (102-159) (150 nM) or aprotinin (0-7.5  $\mu$ M) in buffer containing 0.1 M Tris-HCl (pH 8.0), and 0.05% triton X-100. After 5 min at 37°C, AAPM-AMC (500  $\mu$ M or 1000  $\mu$ M) was added and the fluorescence measured over a two-minute period. Ki values were determined from Dixon plots of the form  $1/V$  versus  $[I]$  performed at two different substrate concentrations (Dixon et al., 1979).

The inhibition of human tissue kallikrein by aprotinin, placental bikunin fragment (7-64) or placental bikunin fragment (102-159) was measured by the incubation of 0.35 nM human tissue kallikrein with placental bikunin (7-64) (0-40 nM) or placental bikunin (102-159) (0-2.5 nM), or aprotinin (0-0.5 nM) in a 1 ml reaction volume containing 50 mM Tris-HCl buffer pH 9.0, 50 mM NaCl, and 0.1% triton x-100. After 5 min. at 37°C, 5 ul of 2 mM PFR-AMC was added achieving 10  $\mu$ M final and the change in fluorescence monitored. The Km for PFR-AMC with human tissue kallikrein under the conditions employed was 5.7  $\mu$ M. The inhibition of human factor Xa (American Diagnostica, Inc, Greenwich, CT) by synthetic placental bikunin (102-159), recombinant placental bikunin, and aprotinin was measured by the incubation of 0.87 nM human factor Xa with increasing amounts of inhibitor in buffer containing 20 mM Tris (pH 7.5), 0.1 M NaCl, and 0.1% BSA. After 5 min. at 37°C, 30 ul of 20 mM LGR-AMC (Sigma) was added and the change in fluorescence monitored. The inhibition of human urokinase (Sigma) by Kunitz inhibitors was measured by the incubation of urokinase (2.7 ng) with inhibitor in a total volume of 1 ml buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.1% Triton x-100. After 5 min. at 37°C, 35 ul of 20 mM GGR-AMC (Sigma) was added and the change in fluorescence monitored. The inhibition of Factor XIa (from Enzyme Research

Labs, Southbend, IN) was measured by incubating FXIa (0.1 nM) with either 0 to 800 nM placental bikunin (7-64), 0 to 140 nM placental bikunin (102-159) or 0 to 40 uM aprotinin in buffer containing 50 mM Hepes pH 7.5, 100 mM NaCl, 2 mM CaCl<sub>2</sub>, 0.01% triton x-100, and 1% BSA in a total volume of 1 ml. After 5 min at  
5 37 C, 10 ul of 40 mM Boc-Glu(OBzl)-Ala-Arg-AMC (Bachem Biosciences, King of Prussia, PA) was added and the change in fluorescence monitored.

**Results:** A direct comparison of the inhibition profiles of placental bikunin (102-159) and aprotinin was made by measuring their inhibition constants with various proteases under identical conditions. The K<sub>i</sub> values are  
10 listed in Table 3 below.

**Table 3 Ki values for the inhibition of various proteases by bikunin (102-159)**

TABLE 3				
Protease (concentration)	bikunin (102-159) Ki (nM)	Aprotinin Ki (nM)	Substrate (concentration)	Km (mM)
Trypsin (48.5 pM)	0.4	0.8	GPK-AMC (0.03 mM)	0.022
Chymotrypsin (5 nM)	0.24	0.86	AAPF-pNA (0.08 mM)	0.027
Bovine Pancreatic Kallikrein (92.0 pM)	0.4	0.02	PFR-AMC (0.1 mM)	0.08
Human Plasma Kallikrein (2.5 nM)	0.3	19.0	PFR-AMC (0.3 mM)	0.46
Human Plasmin (50 pM)	1.8	1.3	GPK-AMC (0.5 mM)	0.73
Human Neutrophil Elastase (19 nM)	323.0	8500.0	AAPM-AMC (1.0 μM)	1.6
Factor XIIa	>300.0	12,000.0	PFR-AMC (0.2 μM)	0.35
Human Tissue Kallikrein (0.35 nM)	0.13	0.004	PFR-AMC (10 μM)	0.0057
factor Xa (0.87 nM)	274	N.I. at 3 μM	LGR-AMC (0.6 mM)	N.D.
urokinase	11000	4500	GGR-AMC (0.7 mM)	N.D.
factor XIa (0.1 nM)	15	288	E(OBz)AR-AMC (0.4 mM)	0.46

Placental bikunin (102-159) and aprotinin inhibit bovine trypsin and 5 human plasmin to a comparable extent under the conditions employed. Aprotinin inhibited elastase with a Ki of 8.5 μM. Placental bikunin (102-159) inhibited elastase with a Ki of 323nM. The K<sub>i</sub> value for the placental bikunin (102-159) inhibition of bovine pancreatic kallikrein was 20-fold higher than that of aprotinin inhibition. In contrast, placental bikunin (102-159) is a more potent 10 inhibitor of human plasma kallikrein than aprotinin and binds with a 56-fold higher affinity.

Because placental bikunin (102-159) is greater than 50 times more potent than Trasylol® as an inhibitor of kallikrein, smaller amounts of human placental bikunin, or fragments thereof (i.e. placental bikunin (102-159)) are needed than 15 Trasylol® in order to maintain the effective patient doses of inhibitor in KIU.

This reduces the cost per dose of the drug and reduces the likelihood of adverse nephrotoxic effects upon re-exposure of the medicament to patients.

Furthermore, the protein is human derived, and thus much less immunogenic in man than aprotinin which is derived from cows. This results in significant 20 reductions in the risk of incurring adverse immunologic events upon re-exposure of the medicament to patients.

**Example 4**

**In vitro specificity of functional placental bikunin fragment (7-64)**

5 In vitro specificity of functional human placental bikunin (7-64) was determined using the materials and methods as described in the Examples above.

10 **Results:** The table below shows the efficacy of placental bikunin (7-64) as an inhibitor of various serine proteases *in vitro*. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159), or aprotinin (Trasylol®).

**Table 4 A Ki values for the inhibition of various proteases by bikunin(7-64)**

TABLE 4A	bikunin(7-64) (concentration)	Aprotinin Ki (nM)	Bikunin (102-159) Ki (nM)
Trypsin (48.5 pM)	0.17	0.8	0.4
Bovine Pancreatic Kallikrein (92.0 pM)	0.4	0.02	0.4
Human Plasma Kallikrein (2.5 nM)	2.4	19.0	0.3
Human Plasmin (50 pM)	3.1	1.3	1.8
Bovine chymotrypsin (5 nM)	0.6	0.9	0.2
Factor XIIa	>300	12000	>300
elastase	>100	8500	323

15 The results show that the amino acid sequence encoding placental bikunin (7-64) can be refolded to obtain an active serine protease inhibitor that is effective against at least four trypsin-like serine proteases.

20 Table 4B below also shows the efficacy of refolded placental bikunin (7-64) as an inhibitor of various serine proteases *in vitro*. Refolded placental bikunin (7-64) was prepared from protein that was certain to be completely deprotected prior to purification and refolding. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159), or aprotinin (Trasylol®).

**Table 4B** Ki values for the inhibition of various proteases by refolded bikunin (7-64)

Protease (concentration)	bikunin (7-64) Ki (nM)	Aprotinin Ki (nM)	bikunin (102-159) Ki (nM)
Trypsin (50 pM)	0.2	0.8	0.3
Human Plasma Kallikrein (0.2 nM)	0.7	19.0	0.7
Human Plasmin (50 pM)	3.7	1.3	1.8
Factor XIIa	not done	12,000	4,500
Factor Xla (0.1 nM)	200	288	15
Human Tissue Kallikrein	2.3	0.004	0.13

Surprisingly, placental bikunin (7-64) was more potent than aprotinin at inhibiting human plasma kallikrein, and at least similar in efficacy as a plasmin inhibitor. These data show that placental bikunin (7-64) is at least as effective as aprotinin, using *in vitro* assays, and that one would expect better or similar potency *in vivo*.

### Example 5

## Expression of placental bikunin variant (102-159) in yeast

The DNA sequence encoding placental bikunin 102-159 (SEQ ID NO.: 6) was generated using synthetic oligonucleotides. The final DNA product

15 consisted (5' to 3') of 15 nucleotides from the yeast  $\alpha$ -mating factor propeptide sequence fused to the in-frame cDNA sequence encoding placental bikunin (102-159), followed by an in-frame stop codon. Upon cloning into a yeast expression vector pS604, the cDNA would direct the expression of a fusion protein comprising an N-terminal yeast  $\alpha$ -mating factor propeptide fused to the 58 amino acid sequence of placental bikunin (102-159). Processing of this fusion protein at a KEX-2 cleavage site at the junction between the  $\alpha$ -mating factor and Kunitz domain was designed to liberate the Kunitz domain at its native N-terminus.

25 A 5' sense oligonucleotide of the following sequence and containing a HindIII site for cloning was synthesized:

GAA GGG GTA AGC TTG GAT AAA AGA TAT GAA GAA TAC TGC ACC GCC  
AAC GCA GTC ACT GGG CCT TGC CGT GCA TCC TTC CCA CGC TGG TAC  
TTT GAC GTG GAG AGG (SEQ ID NO.: 42)

A 3' antisense oligonucleotide of the following sequence and containing both a BamHI site for cloning and a stop codon was synthesized:

5 CGC GGA TCC CTA CTG GCG GAA GCA GCG GAG CAT GCA GGC CTC CTC  
AGA GCG GTA GCT GTT CTT ATT GCC CCG GCA GCC TCC ATA GAT GAA  
GTT ATT GCA GGA GTT CCT CTC CAC GTC AAA GTA CCA GCG  
(SEQ ID NO.: 43)

10 The oligonucleotides were dissolved in 10 mM Tris buffer pH 8.0 containing 1 mM EDTA, and 12 ug of each oligo were added combined and brought to 0.25M NaCl. To hybridize, the oligonucleotides were denatured by boiling for 5 minutes and allowed to cool from 65°C to room temp over 2 hrs. Overlaps were extended using the Klenow fragment and digested with HindIII  
15 and BamHI. The resulting digested double stranded fragment was cloned into pUC19 and sequence confirmed. A clone containing the fragment of the correct sequence was digested with BamHI/HindIII to liberate the bikunin containing fragment with the following + strand sequence:

20 GAA GGG GTA AGC TTG GAT AAA AGA TAT GAA GAA TAC TGC ACC GCC  
AAC GCA GTC ACT GGG CCT TGC CGT GCA TCC TTC CCA CGC TGG TAC  
TTT GAC GTG GAG AGG AAC TCC TGC AAT AAC TTC ATC TAT GGA GGC  
TGC CGG GGC AAT AAG AAC AGC TAC CGC TCT GAG GAG GCC TGC ATG  
CTC CGC TGC TTC CGC CAG TAG GGA TCC (SEQ ID.: 44)

25 which was then gel purified and ligated into BamHI/HindIII cut pS604. The ligation mixture was extracted into phenol/chloroform and purified over a S-200 minispin column. The ligation product was directed transformed into yeast strains SC101 and WHL341 and plated on ura selection plates. Twelve colonies  
30 from each strain were re-streaked on ura drop out plates. A single colony was inoculated into 2 ml of ura DO media and grown over night at 30°C. Cells were pelleted for 2 minutes at 14000x g and the supernatants evaluated for their content of placental bikunin (102-159).

35 *Detection of expression of placental bikunin (102-159) in transformed yeast*

Firstly, the supernatants (50 ul per assay) were evaluated for their capacity to inhibit the *in vitro* activity of trypsin using the assay methods as described in Example 1 (1 ml assay volume). An un-used media only sample as well as a yeast clone expressing an inactive variant of aprotinin served as negative controls. A yeast clone expressing natural aprotinin served as a positive

control and is shown for comparison.

The second method to quantify placental bikunin (102-159) expression exploited use of polyclonal antibodies (pAbs) against the synthetic peptide to monitor the accumulation of the recombinant peptide using Western blots. These 5 studies were performed only with recombinants derived from strain SC101, since these produced greater inhibitory activity than recombinants derived from strain WHL341.

To produce the pAb, two 6-8 week old New Zealand White female rabbits (Hazelton Research Labs, Denver, Pa) were immunized on day zero with 250 ug 10 of purified reduced synthetic placental bikunin (102-159), in Complete Freund's adjuvant, followed by boosts on days 14, 35 and 56 and 77 each with 125 ug of the same antigen in Incomplete Freund's adjuvant. Antiserum used in the present studies was collected after the third boost by established procedures. Polyclonal antibodies were purified from the antiserum over protein A.

15 Colonies 2.4 and 2.5 from transformation of yeast SC101 (Figure 8) as well as an aprotinin control were grown overnight in 50 ml of ura DO media at 30°C. Cells were pelleted and the supernatant concentrated 100-fold using a Centriprep 3 (Amicon, Beverly, MA) concentrator. Samples of each (30 µl) were subjected to SDS-PAGE on 10-20% tricine buffered gels (Novex, San Diego, CA) 20 using the manufacturers procedures. Duplicate gels were either developed with a silver stain kit (Integrated Separation Systems, Nantick, MA) or transferred to nitrocellulose and developed with the purified polyclonal antibody elicited to synthetic bikunin (102-159). Alkaline-phosphatase conjugated goat anti-rabbit antibody was used as the secondary antibody according to the manufacturer's 25 directions (Kirkegaard and Perry, Gaithersburg, MD).

#### *Purification of placental bikunin (102-159) from a transformed strain of SC101*

Fermentation broth from a 1L culture of SC101 strain 2.4 was harvested by centrifugation (4,000 g x 30 min.) then applied to a 1.0 ml column of 30 anhydrochymotrypsin-sepharose (Takara Biochemical Inc., CA), that was previously equilibrated with 50 mM Hepes buffer pH 7.5 containing 0.1M NaCl, 2 mM CaCl<sub>2</sub> and 0.01% (v/v) triton X-100. The column was washed with the same buffer but containing 1.0 M NaCl until the A280nm declined to zero, whereupon the column was eluted with 0.1M formic acid pH 2.5. Eluted 35 fractions were pooled and applied to a C18 column (Vydac, 5µm, 4.6 x 250 mm) previously equilibrated with 0.1% TFA, and eluted with a 50 min. linear gradient of 20 to 80% acetonitrile in 0.1% TFA. Fractions containing placental bikunin

(102-159) were pooled and re-chromatographed on C18 employing elution with a linear 22.5 to 50% acetonitrile gradient in 0.1% TFA.

**Results.** Figure 8 shows the percent trypsin activity inhibited by twelve colonies derived from the transformation of each of strains SC101 and WHL341. The results show that all twelve colonies of yeast strain SC101 transformed with the trypsin inhibitor placental bikunin (102-159) had the ability to produce a substantial amount of trypsin inhibitory activity compared to the negative controls both of which showed no ability to inhibit trypsin. The activity is therefore related to the expression of a specific inhibitor in the placental bikunin variant (102-159) transformed cells. The yeast WHL341 samples contained minimal trypsin inhibitory activity. This may be correlated to the slow growth observed with this strain under the conditions employed.

Figure 9 shows the SDS-PAGE and western analysis of the yeast SC101 supernatants. Silver stained SDS-PAGE of supernatants derived from recombinant yeasts 2.4 and 2.5 expressing placental bikunin (102-159) as well as from the yeast expressing aprotinin yielded a protein band running at approximated 6 kDa, corresponding to the size expected for each recombinant Kunitz inhibitor domain. Western analysis showed that the 6 kDa bands expressed by stains 2.4. and 2.5 reacted with the pAb elicited to placental bikunin (102-159). The same 6 kDa band in the aprotinin control did not react with the same antibody, demonstrating the specificity of the antibody for the placental bikunin variant (102-159).

The final preparation of placental bikunin C-terminal domain was highly pure by silver-stained SDS-PAGE (Figure 10). The overall recovery of broth-derived trypsin inhibitory activity in the final preparation was 31%. N-terminal sequencing of the purified inhibitor indicated that 40% of the protein is correctly processed to yield the correct N-terminus for placental bikunin (102-159) while about 60 % of the material contained a portion of the yeast  $\alpha$ -mating factor. The purified material comprised an active serine protease inhibitor exhibiting an apparent  $K_i$  of 0.35 nM for the *in vitro* inhibition of plasma kallikrein.

In conclusion, the accumulation both of a protease inhibitor activity and a protein immunochemically related to synthetic bikunin (102-159) in fermentation broth as well as the isolation of placental bikunin (102-159) from one of the transformed lines provided proof of expression of placental bikunin in the recombinant yeast strains described herein, showing for the first time the utility of yeasts for the production of placental bikunin fragments.

Additional constructs were prepared in an effort to augment the expression level of the Kunitz domain contained within placental bikunin 102-159, as well as to increase the yield of protein with the correct N-terminus. We hypothesized that the N-terminal residues of placental bikunin 102-159 (YEEY--) may have presented a cleavage site that is only poorly recognized by the yeast KEX-2 protease that enzymically removes the yeast a-factor pro-region. Therefore, we prepared yeast expression constructs for the production of placental bikunin 103-159 (N-terminus of EEY...), 101-159 (N-terminus of NYEEY...) and 98-159 (DMFNYEEY..) in order to modify the P' subsites surrounding the KEX-2 cleavage site. To attempt to augment the levels of recombinant protein expression, we also used the yeast preferred codons rather than mammalian preferred codons in preparing some of the constructs described below. The constructs were essentially prepared as described above for placental bikunin 102-159 (defined as construct #1) but with the following modifications:

15                   Construct #2         placental bikunin 103-159, yeast codon usage  
                        A 5' sense oligonucleotide  
  
20                   GAAGGGTAA GCTTGGATAA AAGAGAAGAA TACTGTACTG CTAATGCTGT  
                        TACTGGTCCA TGTAGAGCTT CTTTCCAAG ATGGTACTTT GATGTTGAAA GA  
                        (SEQ ID NO.: 55)  
  
                        and 3' antisense oligonucleotide  
  
25                   ACTGGATCCT CATTGGCGAA AACATCTCAA CATAACAGGCT TCTTCAGATC  
                        TGTAAGAATT TTTATTACCT CTACAACCAC CGTAAATAAA ATTATTACAA  
                        GAATTCTTT CAACATCAAA GTACCACATCT (SEQ ID NO.: 56)  
  
                        were manipulated as described for the production of an expression construct  
30                   (construct #1 above) for the expression of placental bikunin 102-159

35                   Construct #3         placental bikunin 101-159, yeast codon usage  
                        A 5' sense oligonucleotide  
  
                        GAAGGGTAA GCTTGGATAA AAGAAATTAC GAAGAATACT GTACTGCTAA  
                        TGCTGTTACT GGTCCATGTA GAGCTTCTTT TCCAAGATGG TACTTGATG

TTGAAAGA (SEQ ID NO.: 57)

and the same 3' antisense oligonucleotide as used for construct #2, were manipulated as described for the production of an expression construct (construct #1  
5 above) for the expression of placental bikunin 102-159.

Construct #4      placental bikunin 98-159, yeast codon usage  
A 5' sense oligonucleotide

10 GAAGGGGTAA GCTTGGATAA AAGAGATATG TTTAATTACG AAGAATACTG  
TACTGCTAAT GCTGTTACTG GTCCATGTAG AGCTTCTTT CCAAGATGGT  
ACTTGATGT TGAAAGA (SEQ ID NO.: 58)

15 and the same 3' antisense oligonucleotide as used for construct #2, were manipulated as described for the production of an expression construct (construct #1  
above).

20 Yeast strain SC101 (MAT $\alpha$ , ura 3-52, suc 2) was transformed with the plasmids containing each of the above cDNAs, and proteins were expressed using the methods  
that were described above for the production of placental bikunin 102-159 with  
human codon usage. Approximately 250 ml of each yeast culture was harvested, and  
the supernatant from centrifugation (15 min x 3000 RPM) separately subjected to  
purification over 1 ml columns of kallikrein-sepharose as described above. The  
relative amount of trypsin inhibitory activity in the applicate, the amount of purified  
25 protein recovered and the N-terminal sequence of the purified protein were  
determined and are listed below in Table 7.

Table 7 Relative production levels of different proteins containing the C-terminal Kunitz domain of  
placental bikunin

Construct	Relative conc. of inhibitor in applysate	N-terminal sequencing amount sequence (pmol)	Comments
#2 103-159	none detected	none	no expression
#3 101-159	25 % inhibition	none	low expression
#4 98-159	93 % inhibition	910	DMFNYE- good expression correct product
#1 102-159	82 % inhibition	480	AKEEGV- expression of active incorrectly processed protein

The results show that placental bikunin fragments of different lengths that contain the C-terminal Kunitz domain show wide variation in capacity to express functional secreted protein. Constructs expressing fragments 101-159 and 103-159 yielded little or low enzymic activity in the supernatants prior to purification, and N-terminal sequencing of 0.05 ml aliquots of each purified fraction yielded undetectable amounts of inhibitor. On the other hand expression either of placental bikunin 102-159 or 98-159 yielded significant amounts of protease activity prior to purification. N-terminal sequencing however showed that the purified protein recovered from expression of 102-159 was once again largely incorrectly processed, exhibiting an N-terminus consistent with processing of the majority of the pre-protein at a site within the yeast  $\alpha$ -mating factor pro-sequence. The purified protein recovered from expression of placental bikunin 98-159 however was processed entirely at the correct site to yield the correct N-terminus. Furthermore, nearly twice as much protein was recovered as compared to the recovery of placental bikunin 102-159. Placental bikunin 98-159 thus represents a preferred fragment length for the production of the C-terminal Kunitz domain of placental bikunin by the  $\alpha$ -mating factor pre-pro sequence/ KEX-2 processing system of *S. cerevisiae*,

## Example 6

### Alternative procedure for yeast expression

The 58 amino acid peptide derived from the R74593 translation product can also be PCR amplified from either the R87894-R74593 PCR product cloned into the TA vector™ (Invitrogen, San Diego, CA) after DNA sequencing or from 5 human placental cDNA. The amplified DNA product will consist of 19 nucleotides from the yeast  $\alpha$ -mating factor leader sequence mated to the R74593 sequence which codes for the YEEY--CFRQ (58 residues) so as to make the 10 translation product in frame, constructing an  $\alpha$ -mating factor/Kunitz domain fusion protein. The protein sequence also contains a kex 2 cleavage which will liberate the Kunitz domain at its native N-terminus.

The 5' sense oligonucleotide which contains a HindIII site for cloning will contain the following sequence:

15 GCCAAGCTTG GATAAAAGAT ATGAAGAAT ACTGCACCGC CAACGCA  
(SEQ ID NO.: 30)

The 3' antisense oligonucleotide contains a BamHI site for cloning as well 20 as a stop codon and is of the following sequence:

GGGGATCCTC ACTGCTGGCG GAAGCAGCGG AGCAT (SEQ ID NO.: 31)

The full 206 nucleotide cDNA sequence to be cloned into the yeast 25 expression vector is of the following sequence:

CCAAGCTTGG ATAAAAGATA TGAAGAACAC TGCACCGCCA ACGCAGTCAC  
TGGGCCTTGC CGTGCATCCT TCCCACGCTG GTACTTTGAC GTGGAGAGGA  
ACTCCTGCAA TAACTTCATC TATGGAGGCT GCCGGGGCAA TAAGAACAGC  
TACCGCTCTG AGGAGGCCCTG CATGCTCCGC TGCTTCCGCC AGCAGTGAGG  
30 ATCCCC (SEQ ID NO.: 32)

After PCR amplification, this DNA will be digested with HindIII, BamHI and cloned into the yeast expression vector pMT15 (see US patent 5,164,482, incorporated by reference in the entirety) also digested with HindIII and BamHI. 35 The resulting plasmid vector is used to transform yeast strain SC 106 using the methods described in US patent 5,164,482. The URA 3+ yeast transformants are isolated and cultivated under inducing conditions. The yield of recombinant Placental bikunin variants is determined according to the amount of trypsin

inhibitory activity that accumulated in the culture supernatants over time using the *in vitro* assay method described above. Fermentation broths are centrifuged at 9000 rpm for 30 minutes. The supernatant is then filtered through a 0.4 then a 0.2 µm filter, diluted to a conductivity of 7.5 ms, and adjusted to pH 3 with citric acid. The sample is then batch absorbed onto 200 ml of S-sepharose fast flow (Pharmacia) in 50 mM sodium citrate pH 3 and stirred for 60 min. The gel is subsequently washed sequentially with 2 L of each of: 50 mM sodium citrate pH 3.0; 50 mM Tris-HCL pH 9.0; 20 mM HEPES pH 6.0. The washed gel is transferred into a suitable column and eluted with a linear gradient of 0 to 1 M sodium chloride in 20 mM HEPES pH 6.0. Eluted fractions containing *in vitro* trypsin inhibitory activity are then pooled and further purified either by a) chromatography over a column of immobilized anhydrotrypsin (essentially as described in Example 2); b) by chromatography over a column of immobilized bovine kallikrein; or c) a combination of conventional chromatographic steps including gel filtration and / or anion-exchange chromatography.

#### **Example 7**

#### **Isolation and characterization of native human placental bikunin from placenta**

Bikunin protein was purified to apparent homogeneity from whole frozen placenta (Analytical Biological Services, Inc, Wilmington, DE). The placenta (740 gm) was thawed to room temperature and cut into 0.5 to 1.0 cm pieces, placed on ice and washed with 600 ml PBS buffer. The wash was decanted and 240 ml of placenta pieces placed into a Waring blender. After adding 300 ml of buffer consisting of 0.1 M Tris (pH 8.0), and 0.1 M NaCl , the mixture was blended on high speed for 2 min, decanted into 750.0 ml centrifuge tubes, and placed on ice. This procedure was repeated until all material was processed. The combined slurry was centrifuged at 4500 x g for 60 minutes at 4°C. The supernatant was filtered through cheese cloth and the placental bikunin purified using a kallikrein affinity column made by covalently attaching 70 mg of bovine pancreatic kallikrein (Bayer AG) to 5.0 mls of CNBr activated Sepharose (Pharmacia) according to manufacturers instruction. The material was loaded onto the affinity column at a flow rate of 2.0 ml/min and washed with 0.1 M Tris (pH 8.0), 0.1 M NaCl until absorbance at 280 nm of the wash could no longer be detected. The column was further washed with 0.1 M Tris (pH 8.0), 0.5 M NaCl and then eluted with 3 volumes of 0.2 M acetic acid, pH 4.0. Fractions containing kallikrein and trypsin inhibitory (see below) activity were pooled, frozen, and lyophilized. Placental bikunin was further purified by gel-filtration

chromatography using a Superdex 75 10/30 (Pharmacia) column attached to a Beckman System Gold HPLC system. Briefly, the column was equilibrated in 0.1 M Tris, 0.15 M NaCl, and 0.1% Triton X-100 at a flow rate of 0.5 ml/min. The lyophilized sample was reconstituted in 1.0 ml of 0.1 M Tris, pH 8.0 and injected onto the gel-filtration column in 200 µl aliquots. Fractions were collected (0.5 ml) and assayed for trypsin and kallikrein inhibitory activity. Active fractions were pooled, and the pH of the solution adjusted to 2.5 by addition of TFA. The material was directly applied to a Vydac C18 reverse-phase column (5 micron, 0.46 x 25 cm) which had been equilibrated in 20% acetonitrile in 0.1 %TFA.

5 Separation was achieved using a linear gradient of 20 to 80% acetonitrile in 0.1% TFA at 1.0 ml/min over 50 minutes after an initial 20 minute wash at 20% acetonitrile in 0.1% TFA. Fractions (1ml) were collected and assayed for trypsin and kallikrein inhibitory activity. Fractions containing inhibitory activity were concentrated using a speed-vac concentrator (Savant) and subjected to N-terminal sequence analysis.

10

15

*Functional assays for Placental Bikunin:*

Identification of functional placental bikunin was achieved by measuring its ability to inhibit bovine trypsin and human plasma kallikrein. Trypsin inhibitory activity was performed in assay buffer (50 mM Hepes, pH 7.5, 0.1 M NaCl, 2.0 mM CaCl<sub>2</sub>, 0.1% Triton x-100) at room temperature in a 96-well microtiter plate (Perkin Elmer) using Gly-Pro-Lys-Aminomethylcoumarin as a substrate. The amount of coumarin produced by trypsin was determined by measuring the fluorescence (ex = 370 nm, em = 432 nm) on a Perkin-Elmer LS-50B fluorimeter equipped with a plate reader. Trypsin (23 µg in 100 µl buffer) was mixed with 20 µl of the sample to be tested and incubated for 10 minutes at 25°C. The reaction was started by the addition of 50 µl of the substrate GPK-AMC (33 µM final) in assay buffer. The fluorescence intensity was measured and the % inhibition for each fraction was determined by:

20

25

30

$$\% \text{ inhibition} = 100 \times [1 - F_0/F_1]$$

where F<sub>0</sub> is the fluorescence of the unknown and F<sub>1</sub> is the fluorescence of the trypsin only control. Kallikrein inhibitory activity of the fractions was similarly measured using 7.0 nM kallikrein in assay buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 0.1% triton x-100) and 66.0 µM Pro-Phe-Arg-AMC as a substrate.

35

## Determination of the *in vitro* specificity of placental bikunin

The *In vitro* specificity of native human placental bikunin was determined using the materials and methods as described in the preceding examples above.

Placental bikunin was quantified by active site titration against a known

5 concentration of trypsin using GPK-AMC as a substrate to monitor the fraction of unbound trypsin.

## Protein Sequencing

The 1 ml fraction (C18-29 Delaria) was reduced to 300 ml in volume, on a

10 Speed Vac, to reduce the amount of organic solvent. The sample was then loaded onto a Hewlett-Packard miniature biphasic reaction column, and washed with 1 ml of 2% trifluoroacetic acid. The sample was sequenced on a Hewlett-

Packard Model G1005A protein sequencing system using Edman degradation.

Version 3.0 sequencing methods and all reagents were supplied by Hewlett-

Packard. Sequence was confirmed for 50 cycles.

**Results.** Placental Bikunin was purified to apparent homogeneity by sequential kallikrein affinity, gel-filtration, and reverse-phase chromatography (see purification table below):

20 **Table 5 Purification table for native Placental Bikunin (1-179)**

Step	Vol (ml)	OD 280 (/ml)	OD 280	Units <sup>a</sup> (U)	Units/OD 280
Placenta Supernatant	1800.0	41.7	75,060	3,000,000	40.0
Kallikrein Affinity pH 4.0	20.0	0.17	3.36	16,000	4,880
Kallikrein Affinity pH 1.7	10.2	0.45	4.56	12,000	2,630
Superdex 75	15.0	0.0085	0.13	3,191	24,546

<sup>a</sup>One Unit is defined as that amount which inhibits 50% of trypsin activity in a standard assay.

25 The majority of the kallikrein and trypsin inhibitory activity eluted from the kallikrein affinity column in the pH 4.0 elution. Subsequent gel-filtration chromatography (Figure 5) yielded a peak of kallikrein and trypsin inhibitory activity with a molecular weight range of 10 to 40 kDa as judged by a standard curve generated by running molecular weight standards under identical conditions. Reverse-phase C18 chromatography (Figure 6) yielded 4 peaks of

inhibitory activity with the most potent eluting at approximately 30 % acetonitrile. The activity associated with the first peak to elute from C18 (fraction 29) exhibited an amino acid sequence starting with amino acid 1 of the predicted amino acid sequence of placental bikunin (ADRER...; SEQ ID NO.: 1), and was identical to the predicted sequence for 50 cycles of sequencing (underlined amino acids in Figure 3). Cysteine residues within this sequence stretch were silent as expected for sequencing of oxidized protein. The cysteine residues at amino acid positions 11 and 20 of mature placental bikunin were later identified from sequencing of the S-pyridylethylated protein whereupon PTH-pyridylethyl-cysteine was recovered at cycles 11 and 20.

Interestingly, the asparagine at amino acid residue number 30 of the sequence (Figure 3) was silent showing that this site is likely to be glycosylated. Fraction 29 yielded one major sequence corresponding to that of placental bikunin starting at residue #1 (27 pmol at cycle 1) plus a minor sequence (2 pmol) also derived from placental bikunin starting at residue 6 (SIHD...). This shows that the final preparation sequenced in fraction 29 is highly pure, and most likely responsible for the protease inhibitory activity associated with this fraction (Figure 6).

Accordingly, the final preparation of placental bikunin from C18 chromatography was highly pure based on a silver-stained SDS-PAGE analysis (Figure 7), where the protein migrated with an apparent Mr of 24 kDa on a 10 to 20 % acrylamide tricine gel (Novex, San Diego, CA) calibrated with the following molecular weight markers: insulin (2.9 kDa); bovine trypsin inhibitor (5.8 kDa); lysozyme (14.7 kDa);  $\beta$ -lactoglobulin (18.4 kDa); carbonic anhydrase (29 kDa); and ovalbumin (43 kDa). The above size of placental bikunin on SDS-PAGE is consistent with that predicted from the full length coding sequence (Figure 4F).

As expected based on the N-terminal sequencing results described above, the purified protein reacted with an antibody elicited to placental bikunin (7-64) to yield a band with the same Mr (Figure 12A) as observed for the purified preparation detected on gels by silver stain (Figure 7). However, when the same preparation was reacted with an antibody elicited to synthetic placental bikunin (102-159), a band corresponding to the full length protein was not observed. Rather, a fragment that co-migrated with synthetic bikunin (102-159) of approximately 6 kDa was observed. The simplest interpretation of these results is that the purified preparation had undergone degradation subsequent to purification to yield an N-terminal fragment comprising the N-terminal domain and a C-terminal fragment comprising the C-terminal

domain. Assuming that the fragment reactive against antiserum to placental bikunin (7-64) is devoid of the C-terminal end of the full length protein, the size (24 kDa) would suggest a high state of glycosylation.

Table 6. below shows the potency of *in vitro* inhibition of various serine proteases by placental bikunin. Data are compared with that obtained with aprotinin (Trasylol®).

**Table 6 Ki values for the inhibition of various proteases by placental bikunin**

TABLE 6	Placental Bikunin (concentration)	Aprotinin Ki (nM)
Trypsin (48.5 pM)	0.13	0.8
Human Plasmin (50 pM)	1.9	1.3

The results show that placental bikunin isolated from a natural source (human placenta) is a potent inhibitor of trypsin-like serine proteases.

#### **Example 8**

#### **Expression pattern of placental bikunin amongst different human organs and tissues**

A multiple tissue northern was purchased from Clontech which contained 2 µg of polyA+ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Two different cDNA probes were used: 1) a gel purified cDNA encoding placental bikunin (102-159); 2) the 780 base pair PCR-derived cDNA (Figure 4E) liberated from a TA clone by digestion with EcoRI and gel purified. Each probe was labeled using  $^{32}\text{P}$ -dCTP and a random priming labeling kit from Boehringer Mannheim Biochemicals (Indiana), then used to hybridize to the multiple tissue northern according to the manufacturers specifications. Autoradiographs were generated using Biomax film with an 18 hr exposure time, and developed using a Umax Scanner and scanned using Adobe Photoshop.

**Results.** The pattern of tissue expression observed using a placental bikunin (102-159) probe (Figure 11A) or a larger probe containing both Kunitz domains of placental bikunin (Figure 11B) was essentially the same as might be expected. The placental bikunin mRNA was most abundant in pancreas and placenta. Significant levels were also observed in lung, brain and kidney, while lower

levels were observed in heart and liver, and the mRNA was undetectable in skeletal muscle. The transcript size was 1.95 kilobases in all cases, in close agreement with the predicted size of placental bikunin deduced both from EST overlay and cloning of full length cDNA described in preceding sections.

5       The broad tissue distribution of the mRNA shows that placental bikunin is broadly expressed. Since the protein also contains a leader sequence it would have ample exposure to the human immune system, requiring that it become recognized as a self protein. Additional evidence for a broad tissue distribution of placental bikunin mRNA expression was derived from the fact that some of  
10      the EST entries with homology to placental bikunin (Figure 4B) were derived from human adult and infant brain, and human retina, breast, ovary, olfactory epithelium, and placenta. It is concluded therefore that administration of the native human protein to human patients would be unlikely to elicit an immune response.

15      Interestingly, the expression pattern of placental bikunin is somewhat reminiscent of that for bovine aprotinin which is found in high levels in bovine lung and pancreas. To further elucidate the expression pattern of placental bikunin, RT-PCR of total RNA from the following human cells was determined:  
20      un-stimulated human umbilical vein endothelial cells (HUVECs), HK-2 (line derived from kidney proximal tubule), TF-1 (erythroleukemia line) and phorbol ester (PMA)-stimulated human peripheral blood leukocytes. The probes used:

CACCTGATCGCGAGACCCC (sense; SEQ ID NO.: 59);

25      CTGGCGGAAGCAGCGGAGCATGC (antisense; SEQ ID NO.: 60),

were designed to amplify a 600 b.p. placental bikunin encoding cDNA fragment. Comparisons were normalized by inclusion of actin primers to amplify an 800 b.p. actin fragment. Whereas the 800 b.p. fragment identified on agarose gels  
30      with ethidium bromide was of equal intensity in all lanes, the 600 b.p. placental bikunin fragment was absent from the HUVECs but present in significant amounts in each of the other cell lines. We conclude that placental bikunin is not expressed in at least some endothelial cells but is expressed in some leukocyte populations.

35

#### **Example 9**

Purification and properties of Placental Bikunin (1-170) highly purified from a

## Baculovirus / Sf9 expression system

A large fragment of Placental bikunin containing both Kunitz domains (Placental Bikunin 1-170) was expressed in Sf9 cells as follows. Placental bikunin cDNA obtained by PCR (Figure 4E) and contained within a TA vector (see previous Examples) was liberated by digestion with HindIII and XbaI yielding a fragment flanked by a 5' XbaI site and 3' HindIII site. This fragment was gel purified and then cloned into the M13mp19 vector (New England Biolabs, Beverly, MA). In vitro mutagenesis (Kunkel T.A., (1985) Proc. Natl. Acad. Sci. USA, 82: 488-492) was used to generate a PstI site 3' to the XbaI site at the 5' end, but 5' to the sequence encoding the ATG start site, natural placental bikunin signal peptide and mature placental bikunin coding sequence. The oligonucleotide used for the mutagenesis had the sequence:

5' CGC GTC TCG GCT GAC CTG GCC CTG CAG ATG GCG CAC GTG TGC GGG 3'  
(SEQ ID NO.: 61)

15 A stop codon (TAG) and BglII / XmaI site was similarly engineered at the 3' end of  
the cDNA using the oligonucleotide:

5' CTG CCC CTT GGC TCA AAG TAG GAA GAT CTT CCC CCC GGG GGG GTG  
20 GTT CTG GCG GGG CTG 3' (SEQ ID NO.: 62).

The stop codon was in frame with the sequence encoding placental bikunin and caused termination immediately following the Lysine at amino acid residue 170, thus encoding a truncated placental bikunin fragment devoid of the putative transmembrane domain. The product from digestion with PstI and BglII was isolated and cloned into the BacPac8 vector for expression of Placental bikunin fragment (1-170) which contains both Kunitz domains but which is truncated immediately N-terminal to the putative transmembrane segment.

The expression of Bikunin by Sf-9 insect cells was optimal at a multiplicity of infection of 1 to 1 when the medium was harvested at 72 h post infection. After harvesting, the baculovirus cell culture supernatant (2L) was adjusted to pH 8.0 by the addition of Tris-HCl. Bikunin was purified by chromatography using a 5 ml bovine pancreatic kallikrein affinity column as previously described in Example 7 for the purification of native placental bikunin from placenta. Eluted material was adjusted to pH 2.5 with TFA and subjected to chromatography on a C18 reverse-phase column (1.0 x 25 cm) equilibrated in 10% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The bikunin was eluted with a linear gradient of 10 to 80% acetonitrile

in 0.1% TFA over 40 min. Active fractions were pooled, lyophilized, redissolved in 50 mM Hepes (pH 7.5), 0.1 M NaCl, 2 mM CaCl<sub>2</sub>, and 0.1% triton x-100, and stored at -20°C until needed. The concentration of recombinant bikunin was determined by amino acid analysis.

5

**Results.** Recombinant bikunin was purified from baculovirus cell culture supernatant using a 2-step purification protocol as shown below, to yield an active trypsin inhibitor (Table 8 below).

10 **Table 8 Purification of recombinant bikunin from transformed culture supernatant**

TABLE 8

Purification Step	Vol (ml)	OD 280/ml	OD 280 total	Units (U)	Specific activity (U/OD)
Supernatant	2300.0	9.0	20,700	6,150,000	297
Kallikrein affinity	23.0	0.12	2.76	40,700	14,746
C18 reverse-phase	0.4	3.84	1.54	11,111	72,150

Chromatography of the crude material over an immobilized bovine pancreatic kallikrein affinity column selectively isolated 0.013 % of the protein and 0.67 % of the trypsin inhibitory activity present. The majority of the trypsin inhibitory activity present in the starting supernatant did not bind to the immobilized kallikrein and is not related to bikunin (results not shown). Subsequent chromatography using C18 reverse-phase yielded a further purification of 5-fold, with a recovery of 0.2%. The final preparation was highly pure by SDS-PAGE (Figure 13), exhibiting an Mr of 21.3 kDa, and reacted on immunoblots to rabbit anti-placental bikunin 102-159 (not shown). N-terminal sequencing (26 cycles) yielded the expected sequence for mature placental bikunin (Figure 4F) starting at residue +1(ADRER....), showing that the signal peptide was correctly processed in Sf9 cells.

Purified placental bikunin from Sf9 cells (100 pmol) was pyridylethyl-alkylated, CNBr digested and then sequenced without resolution of the resulting fragments. Sequencing for 20 cycles yielded the following N-terminii:

Sequence	Amount	Placental bikunin residue #
----------	--------	-----------------------------

LRCFrQQENPP-PLG-----	21 pmol	154 - 168 (SEQ ID NO.: 63)
ADRERSIHDFCLVSKVVGRG	20 pmol	1 - 20 (SEQ ID NO.: 64)
FNYeEYCTANAVTGPCRASF	16 pmol	100 - 119 (SEQ ID NO.: 65)

Pr--Y-V-dGS-Q-F-Y-G

6 pmol 25 - 43 (SEQ ID NO.: 66)

Thus N-terminii corresponding to each of the expected four fragments were recovered. This confirms that the Sf9 expressed protein contained the entire  
5 ectodomain sequence of placental bikunin (1-170).

#### Example 10

##### Inhibition specificity of purified placental bikunin derived from Sf9 cells.

The *in vitro* specificity of recombinant bikunin was determined using the materials and methods as described in Examples 3, 4 and 7. In addition, the inhibition of human tissue kallikrein by bikunin was measured by the incubation of 0.35 nM human tissue kallikrein recombinant bikunin in buffer containing 50 mM Tris (pH 9.0), 50 mM NaCl, and 0.01% triton x-100. After 5 min. at 37°C, 5 µl of 2 mM PFR-AMC was added and the change in fluorescence monitored.

Inhibition of tissue plasminogen activator (tPA) was also determined as follows: tPA (single chain form from human melanoma cell culture from Sigma Chemical Co, St Louis, MO) was pre-incubated with inhibitor for 2 hr at room temperature in 20 mM Tris buffer pH 7.2 containing 150 mM NaCl, and 0.02% sodium azide. Reactions were subsequently initiated by transfer to a reaction system comprising the following initial component concentrations: tPA (7.5 nM), inhibitor 0 to 6.6 µM, Dile-Lpro-Larg-pNitroaniline (1mM) in 28 mM Tris buffer pH 8.5 containing 0.004 % (v/v) triton x-100 and 0.005% (v/v) sodium azide. Formation of p-Nitroaniline was determined from the A405nm measured following incubation at 37 C for 2hr.

The table below show the efficacy of recombinant bikunin as an inhibitor of various serine proteases *in vitro*. Data is shown compared against data obtained for screening inhibition using either recombinant bikunin, or aprotinin.

**Table 9 Comparisons of Ki values for the inhibition of various proteases by recombinant placental bikunin (1-170) or aprotinin**

TABLE 9

Protease (concentration)	Recombinant Bikunin Ki (nM)	Aprotinin Ki (nM)
Trypsin (48.5 pM)	0.064	0.8
Human Plasma Kallikrein (2.5 nM)	0.18	19.0
Human Tissue Kallikrein (0.35 nM)	0.04	0.004
Bovine Pancreatic Kallikrein (100 pM)	0.12	0.02
Human Plasmin (50 pM)	0.23	1.3
factor Xa (0.87 nM)	180	5% Inhibition at 31 $\mu$ M
factor XIa (0.1 nM)	3.0	288
tissue plasminogen activator (7.5 nM)	< 60	no inhibition at 6.6 $\mu$ M
Tissue Factor VIIa	800	no inhibition at 1 $\mu$ M

5

The results show that recombinant bikunin can be expressed in insect cells to yield an active protease inhibitor that is effective against at least five different serine protease inhibitors. Recombinant bikunin was more potent than aprotinin against human plasma kallikrein, trypsin and plasmin. Surprisingly, the recombinant 10 bikunin was more potent than the synthetically derived bikunin fragments (7-64) and (102-159) against all enzymes tested. These data show that recombinant bikunin is more effective than aprotinin, using *in vitro* assays, and that one would expect better *in vivo* potency.

Besides measuring the potencies against specific proteases, the capacity of 15 recombinant placental bikunin (1-170) to prolong the activated partial thromboplastin time (APTT) was evaluated and compared with the activity associated with aprotinin. Inhibitor was diluted in 20 mM Tris buffer pH 7.2 containing 150 mM NaCl and 0.02% sodium azide and added (0.1 ml) to a cuvette contained within an MLA Electra<sup>R</sup> 800 Automatic Coagulation Timer coagulometer (Medical Laboratory Automation, Inc., 20 Pleasantville, N.Y.). The instrument was set to APTT mode with a 300 sec. activation time and the duplicate mode. Following addition of 0.1 ml of plasma (Specialty Assayed Reference Plasma lot 1-6-5185, Helena Laboratories, Beaumont, TX), the APTT reagent (Automated APTT-lot 102345, from Organon Teknika Corp., Durhan, NC) and 25 mM CaCl<sub>2</sub> were automatically dispensed to initiate clotting, and the 25 clotting time was monitored automatically. The results (Figure 14) showed that a

doubling of the clotting time required approximately 2  $\mu$ M final aprotinin, but only 0.3  $\mu$ M Sf9 derived placental bikunin. These data show that placental bikunin is an effective anticoagulant, and useful as a medicament for diseases involving pathologic activation of the intrinsic pathway of coagulation.

5

### **Example 11**

#### **Measurement of Tracheal Potential Difference in the Guinea-pig**

The aim of this study was to investigate the effect of the Kunitz serine protease inhibitor Bikunin, and the sodium channel blocker amiloride on guinea-pig tracheal potential difference 3 hours post treatment. These agents were delivered into the cephalad trachea by topical instillation. TPD was monitored 2 hours later for 60 minutes. The procedure used in this Example is described in Newton et al. in "Cilia, Mucus and Mucociliary Interactions," Ed., Baum, G.L. et al., Marcel Dekker, New York, 1998; Newton et al., Ped. Pulm. S17, Abs. 364, 1998).

15

#### **Materials and methods/Reagents used**

Aqueous formulations of Bikunin (5 and 50  $\mu$ g/mL (SEQ ID NO: 52)) and amiloride (obtained from Sigma Chemicals, St. Louis, MO, USA)(100  $\mu$ M) were prepared, sterile filtered and endotoxin tested prior to use. These formulations were prepared in Hank's Balanced salt solution (HBSS) and contained 137 mM NaCl, 3 mM KCl, 3 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.2% Tween-80, pH 7.1) was prepared, sterile filtered and endotoxin tested for use in this example. HBSS was used as a control solution. Hypnorm® (Fentanyl citrate 0.315 mg/mL and Fluanisone 10 mg/mL) was obtained from Janssen Animal Health and Hypnovel® (Midazolam 5 mg/mL) was obtained from Roche. Male Dunkin-Hartley guinea pigs (550-750 g) were supplied by David Hall, UK. Thermistor probes were obtained from Kane-May Ltd, UK.

#### **Induction of anaesthesia and administration of Bikunin into tracheal airway**

Animals were anaesthetised using halothane. Once a satisfactory level of anaesthesia was induced a small incision was made below the lower jaw. The trachea was exposed and 100  $\mu$ l volume of vehicle, bikunin (0.5  $\mu$ g or 5  $\mu$ g) or amiloride (100  $\mu$ M) was instilled onto the tracheal surface using a needle and syringe. Once injected, the skin incision was sealed using Vetbond® (cyanocacrylate tissue glue). The animals were then allowed to recover.

35

#### **Preparation of guinea-pig for measurement of tracheal potential difference**

Two hours following agent treatment, guinea-pigs were anaesthetised for a

second time with Hypnom® and Hypnovel® and immobilised in a supine position. Rectal temperature, measured with a thermistor probe was maintained at 37° C by manual adjustment of a heat lamp. A ventral midline incision was made from the lower jaw to the clavicles. Using blunt dissection a length of trachea was exposed and bisected at the upper edge of the sternum. The external jugular vein was exposed and cannulated. The caudal part of the trachea was then cannulated to allow the animal to spontaneously breath room air. The animal was then placed supine and its body temperature maintained using the heat lamp. 20 min. following induction of i.v. anaesthesia the tracheal agar electrode was inserted into the cephalad trachea and tracheal potential difference was measured for 60 minutes. The reference electrode was placed under cephalad trachea in contact with the trachea cartilage. The wound site was covered to prevent drying.

### **Results**

As shown in Figure 15, Bikunin (5 µg) inhibited the potential difference in guinea pig trachea in vivo following three hours of treatment relative to vehicle. The effect of Amiloride (100 µM) and Bikunin (0.5 µg) is shown for comparison.

### **Experiment 12**

#### **The Effect of Bikunin on Tracheal Mucus Velocity in the Guinea-pig**

The aim of this study was to investigate the effect of the Kunitz family serine protease inhibitor Bikunin on guinea-pig tracheal mucus velocity 1.5 hours post treatment. This agent was delivered into the cephalad trachea by topical instillation. TMV was monitored 1.5 hours later for 60 mins. The procedure used in this Example is described in Newton et al. in "Cilia, Mucus and Mucociliary Interactions," Ed., Baum, G.L. et al., Marcel Dekker, New York, 1998; Newton et al., Ped. Pulm. S17, Abs. 364, 1998).

#### **Materials and methods/Reagents used:**

A Bikunin formulation (50 µg/mL Bikunin (SEQ ID NO: 52) was prepared in HBBS containing 137 mM NaCl, 3 mM KCl, 3 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.2% Tween-80, pH 7.1). The formulation was sterile filtered and endotoxin tested prior to use in this example. HBSS was used as a control solution. Hypnorm® (Fentanyl citrate 0.315 mg/mL and Fluanisone 10 mg/mL) was obtained from Janssen Animal Health and Hypnovel® (Midazolam 5 mg/mL) was obtained from Roche. Male Dunkin-Hartley guinea pigs (550-750 g) were supplied by David Hall, UK. Thermistor probes were obtained from Kane-May Ltd, UK.

### *Induction of anaesthesia and administration of Bikunin into tracheal airway*

Animals were anaesthetized using halothane. Once a satisfactory level of anaesthesia was induced, a small incision was made below the lower jaw. The trachea was exposed and 100 ul volume of vehicle or bikunin (5 ug) was instilled onto the tracheal surface using a needle and syringe. Once instilled, the skin incision was sealed using Vetbond® (cyanocacrylate tissue glue). The animals were then allowed to recover.

### *Measurement of tracheal mucus velocity(TMV)*

TMV was monitored using a lead collimated miniature Beta particle detector probe arranged to detect the radioactivity emitted from an injected aliquot of  $^{32}\text{P}$ -labelled *Saccharomyces cerevisiae* as it was transported on the tracheal mucociliary layer of an anaesthetized guinea pig (Newton and Hall 1998) Figure 16(a) illustrates the arrangement of the syringe and beta probe. Figure 16(b) illustrates the counts detected by the probe as the  $^{32}\text{P}$ -labelled *S.cerevisiae* is transported along the tracheal mucociliary layer.

70 minutes following instillation of bikunin, each animal was anaesthetized for a second time using Hyponorm® and Hyponovel® and immobilized in a supine position. The first TMV measurement was made 20 minutes afterwards. Subsequent measurements were taken every 15 minutes. The procedure for TMV measurements is described, in detail, in Newton et al., "Cilia. Mucus and Mucociliary Interactions." Ed. Baum, G.L., Preil, Z., Roth, Y., Liron., Ostfield, E., Marcel Dekker. New York, 1990 and Newton et al. in *Pediatric Pulmonology* S17, Abs 364, 1998.

### **25 Results**

As shown in Figure 16(c), Bikunin (5 ug) increased TMV in vivo in guinea pig, relative to saline, over a sustained period of 1.5 to 2.5 hours following administration.

### **Experiment 13**

**30 Bikunin decreases sodium current in cultured human bronchial epithelial (HBE) cell short circuit current (Isc) in vitro.**

Tertiary HBE cell monolayers grown to confluence were mounted in modified Ussing chambers, immersed in Krebs buffer (KBR) solution and bubbled with 95%O<sub>2</sub>/5%CO<sub>2</sub> warmed to 37C.

**35 Cells were left to equilibrate for 20 minutes before calibrating for background noise and fluid resistance. Transepithelial potential difference was then clamped to 0 mV using a WPI EVC 4000 voltage clamp. Ag/AgCl electrodes were used to monitor**

Isc. Once a stable baseline was achieved (typically 10-20 min), cells were treated with amiloride (10 uM). Once a response to amiloride was seen, it was washed out with KBR solution. After return to baseline and equilibration, Bikunin (0.5-50 ug/mL in PBS) or PBS control was added. 90 minutes following agent treatment, amiloride (10 uM) was added. Once the current was stable forskolin (10 uM in PBS) and then bumetanide (100 uM in PBS) was added.

## Results

As shown in Figure 17, Bikunin (70 nM) inhibited sodium current in vitro in 10 human bronchial epithelial cells over a 90 minute period. Forskolin induced cAMP-mediated chloride secretion and monolayer resistance was unaffected.

### Experiment 14

#### The effect of hypertonic saline (14.4%) on TMV in the guinea pig

15 The aim of this comparative study was to investigate the effect of hypertonic saline (14.4% x 5 min) on guinea-pig tracheal mucus velocity. This agent was delivered into the cephalad trachea by aerosol. TMV was monitored immediately and every 15 minutes for 30 minutes. The procedure used in this Example is described in Newton et al. in "Cilia, Mucus and Mucociliary Interactions," Ed., Baum, 20 G.L. et al., Marcel Dekker, New York, 1998; Newton et al., Ped. Pulm. S17, Abs. 364, 1998).

#### Materials and methods/Reagents used

25 Hypnorm® (Fentanyl citrate 0.315 mg/mL and Fluanisone 10 mg/mL) was obtained from Janssen Animal Health and Hypnovel® (Midazolam 5 mg/mL) was obtained from Roche. Male Dunkin-Hartley guinea pigs (550-750 g) were supplied by Harlan UK Ltd. Thermistor probes were obtained from Kane-May Ltd, UK.

#### Measurement of tracheal mucus velocity:

30 Animals were anaesthetized using Hypnorm® and Hypnovel®. TMV was monitored using a lead collimated miniature Beta particle detector probe arranged to detect the radioactivity emitted from an injected aliquot of <sup>32</sup>P-labelled *Saccharomyces cerevisiae* as it was transported on the tracheal mucociliary layer of an anaesthetized guinea pig (Newton and Hall 1998)

35 The first TMV measurement (run 1) was made 20 minutes after administration. Subsequent measurements were taken every 15 minutes. At a time point 6 minutes before the second run, a 5 minute aerosol of saline (0.9%) or hypertonic saline (14.4%)

was administered. The radiolabelled tracer particles were given via the 0.5 um hole made in the tracheae. An aerosol of ether saline (0.9%) or hypertonic saline (14.4%) was generated by a Pari pressure nebulizer. The aerosol was switched off one minute before the second run. The procedure for TMV measurements is described, in detail,  
5 in Newton et al., "Cilia, Mucus and Mucociliary Interactions." Ed. Baum, G.L., Preil, Z., Roth, Y., Liron., Ostfield, E., Marcel Dekker. New York, 1990 and Newton et al. in *Pediatric Pulmonology* S17, Abs 364, 1998.

### **Results**

10 As shown in Figure 18, hypertonic saline (14.4% x 5 mins) caused a transient increase in TMV immediately after aerosol.

### **Experiment 15**

#### **Effect of Amiloride on TMV in the Guinea-pig**

15 The aim of this study was to investigate the effect of amiloride (10mM x 20 min.) on guinea-pig tracheal mucus in the anaesthetized spontaneously breathing guinea pig. This agent was delivered into the cephalad trachea by aerosolization as described in Example 14. The TMV measurement procedure used in this Example is described in Newton et al. in "Cilia, Mucus and Mucociliary Interactions," Ed., Baum,  
20 G.L. et al., Marcel Dekker, New York, 1998; Newton et al., Ped. Pulm. S17, Abs. 364, 1998).

#### **Materials and methods/Reagents used**

An amiloride formulation (10 mM) in water was prepared for this example.

25 Hypnorm® (Fentanyl citrate 0.315 mg/mL and Fluanisone 10 mg/mL) was obtained from Janssen Animal Health and Hypnovel® (Midazolam 5 mg/mL) was obtained from Roche. Male Dunkin-Hartley guinea pigs (550-750 g) were supplied by Harlan UK Ltd. Thermistor probes were obtained from Kane-May Ltd, UK.

#### **30 Measurement of trachael mucus velocity**

Animals were anaesthetized using Hypnorm® and Hypnovel®. TMV was monitored using a lead collimated miniature Beta particle detector probe arranged to detect the radioactivity emitted from an injected aliquot of <sup>32</sup>P-labelled *Saccharomyces cerevisiae* as it was transported on the tracheal mucociliary layer of an anaesthetized  
35 guinea pig. Guinea pigs were anaesthetized with Hypnorm® and Hyponovel at time 0. Amiloride (10mM x 20 min) was administered with Hyponorm® and Hypnovel at t = 0. Amiloride (10 mM x 20 min) was administered by aerosol. The first TMV

measurement was made immediately afterwards and subsequent measurements were taken every 15 minutes.

### **Results**

5 As shown in Figure 19, amiloride (10 mM x 20 mins) caused a statistically significant increase in TMV 15 minutes after aerosol.

### **Example 16**

10 **Aprotinin double mutein decreases sodium current in cultured human bronchial epithelial (HBE) cell short circuit current (Isc)**

The aim of this study was to investigate the effect of the Kunitz family serine protease inhibitor Aprotinin double mutein on Isc in vitro. Tertiary HBE cell monolayers grown to confluence were mounted in modified Ussing chambers, immersed in Krebs buffer (KBR) solution and bubbled with 95% O<sub>2</sub>/5%CO<sub>2</sub> warmed to 37C. Aprotinin double mutein is Des Pro2-Ser10-Arg15-Asp24-Thr26-Glu31-Asn41-Glu53-Aprotinin which is described in Example 1 of EP 821 007, published January 28, 1998, incorporated by reference in its entirety.

15 Cells were left to equilibrate for 20 minutes before calibrating for background noise and fluid resistance. Transepithelial potential difference was then clamped to 0 mV using a WPI EVC 4000 voltage clamp. Ag/AgCl electrodes were used to monitor Isc. Once a stable baseline was achieved (typically 10-20 mins), cells were treated with a amiloride (10uM). Once a response to amiloride was seen, it was washed out with KBR solution. After return to baseline and equilibration, Bikunin (5 ug/mL), Aprotinin double 20 mutein (0.5 to 5 ug/mL), Aprotinin (1.5 to 5 ug/mL) or PBS was added. 90 minutes following agent treatment, amiloride (10uM) was added.

### **Results**

25 As shown in Figure 20, Aprotinin double mutein (0.5 to 5 ug/mL) dose dependently inhibited sodium current in vitro in human bronchial epithelial cells over a 90 minute period.

Although certain embodiments of the invention have been described in detail for the purpose of illustration, it will be readily apparent to those skilled in the art that the methods and formulations described herein may be modified 30 without departing from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

**WE CLAIM:**

1. A method for accelerating the rate of mucociliary clearance in a subject in need of such treatment comprising administering to the subject an effective mucociliary clearance stimulatory amount of a composition comprising a Kunitz-type serine protease inhibitor and a physiologically acceptable carrier.  
5
2. The method according to claim 1, wherein the composition is administered to the lung airways.
3. The method according to claim 1, wherein said composition is  
10 administered directly by aerosolization.
4. The method according to claim 1, wherein said composition is administered directly as an aerosol suspension into the mammal's respiratory tract.
5. The method according to claim 4, wherein said aerosol suspension  
15 includes respirable particles ranging in size from about 1 to about 10 microns.
6. The method according to claim 4, wherein said aerosol suspension includes respirable particles ranging in size from about 1 to about 5 microns.
7. The method according to claim 4, wherein said aerosol suspension is delivered to said subject by a pressure driven nebulizer.  
20
8. The method according to claim 4, wherein said aerosol suspension is delivered to said subject by an ultrasonic nebulizer.
9. The method according to claim 4, wherein said aerosol suspension is delivered to said subject by a non-toxic propellant.
10. The method according to claim 1, wherein said carrier is a member  
25 selected from the group consisting of a physiologically buffered solution, an isotonic saline, normal saline, and combinations thereof.
11. The method according to claim 1 wherein the Kunitz-type serine protease inhibitor is aprotinin.
12. The method according to claim 1, wherein the Kunitz-type serine  
30 protease inhibitor comprises the amino acid sequence:

MAQLCGL RRSRAFLALL GSLLLGVLA -1

ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50

YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100

NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150

35 ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200

QERALRTVWS SGDDKEQLVK NTYVL 225

(SEQ ID NO.: 49).

13. The method according to claim 1, wherein the Kunitz-type serine protease inhibitor comprises the amino acid sequence:

		AGSFLAWL GSLLLGVLA -1
5	ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50	
	YLTKEECLKK CATVTENATG DLATTSRNAAD SSVPSPARRQ DSEDHSSDMF 100	
	NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150	
	ACMLRCFROO ENPPLPLGSK VVLAGAVS 179	

(SEQ ID NO.: 2),

10	MLR AEADGVSRLL GSLLLGVLA -1
	<b>ADRERSIHDF CLVSKVVGRG RASMPRWYN VTDGSCQLFV YGGCDGNSNN</b> 50
	<b>YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF</b> 100
	<b>NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE</b> 150
15	<b>ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN</b> 200
	<b>QERALRTVWS SGDDKEQLVK NTYVL</b> 225
	(SEQ ID NO.: 45),

MAQECGL KRSKATFALL CSELESQVLT  
20 ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100  
NYEEYCTANA VTGPCRASFV RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200  
QERALRTVWS FGD 213  
25 (SEQ ID NO.: 47),

ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSQLFV YGGCDGNSNN 50  
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF 100  
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
30 ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200  
QERALRTVWS SGDDKEQLVK NTYVL 225  
(SEQ ID NO.: 70),

and

35

**ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN** 50  
**YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRO DSEDHSSDMF** 100

NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200  
QERALRTVWS FGD 213  
(SEQ ID NO.: 71).

5

14. The method according to claim 1, wherein the Kunitz-type serine protease inhibitor comprises the amino acid sequence:

10 IHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKKECLKK CATV 64  
(SEQ ID NO.: 4),  
  
15 CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKKECLKK C 61  
(SEQ ID NO.: 5),  
  
20 YEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
ACMLRCFRQ 159  
(SEQ ID NO.: 6),  
  
25 CTANAVTGPC RASFPRWYFD VERNSCNNFI YGGCRGNKNS YRSEE 150  
ACMLRC 156  
(SEQ ID NO.: 7),  
30 IHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKKECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 75  
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 125  
ACMLRCFRQ 159  
(SEQ ID NO.: 3),  
  
35 CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKKECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100  
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
ACMLRC 156  
(SEQ ID NO.: 50),

ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 25

YLTKKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 75  
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 125  
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS 179  
(SEQ ID NO.: 1),

5

and

ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN

50

10 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF  
100

NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE  
150

ACMLRCFRQQ ENPPLPLGSK 170

15 (SEQ ID NO.: 52).

15. The method according to claim 1, wherein the Kunitz-type serine protease inhibitor comprises the amino acid sequence:

20 ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DS 92  
(SEQ ID NO.: 8).

25 16. The method according to claims 12, 13, 14 or 15, wherein the Kunitz-type serine protease inhibitor is glycosylated.

17. The method according to claims 12, 13, 14 or 15, wherein the Kunitz-type serine protease inhibitor contains at least one intra-chain cysteine-cysteine disulfide bond.

30

18. The method according to claims 12, 13, 14, or 15, wherein the Kunitz-type serine protease inhibitor contains at least one intra-chain cysteine-cysteine disulfide bond selected from the cysteine-cysteine paired groups consisting of CYS11-CYS61, CYS20-CYS44, CYS36-CYS57, CYS106-CYS156, CYS115-CYS139, 35 and CYS131-CYS152, wherein the cysteine residues are numbered according to the amino acid sequence of native human placental bikunin.

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19. Use of a Kunitz-type serine protease inhibitor in manufacturing a medicament for accelerating the rate of mucociliary clearance in a subject in need of such treatment.
20. Use according to claim 19, wherein the Kunitz-type serine protease inhibitor is in a form suitable for administration to lung airways.
- 5 21. Use according to claim 19, wherein the Kunitz-type serine protease inhibitor is in a form suitable for administration by aerosolization.
22. Use according to claim 19, wherein the Kunitz-type serine protease inhibitor is in a form suitable for administration as an aerosol suspension into
- 10 the mammal's respiratory tract.
23. Use according to claim 22, wherein said aerosol suspension includes respirable particles ranging in size from about 1 to about 10 microns.
24. Use according to claim 22, wherein said aerosol suspension includes respirable particles ranging in size from about 1 to about 5 microns.
- 15 25. Use according to claim 22, wherein said aerosol suspension is generated by pressure driven nebulizer.
26. Use according to claim 22, wherein said aerosol suspension is generated by an ultrasonic nebulizer.
27. Use according to claim 22, wherein said aerosol suspension includes a non-toxic propellant.
- 20 28. Use according to claim 19, wherein medicament includes a carrier selected from the group consisting of a physiologically buffered solution, an isotonic saline, normal saline, and combinations thereof.
29. Use according to claim 19, wherein the Kunitz-type serine protease inhibitor is aprotinin.
- 25 31. Use according to claim 19, wherein the Kunitz-type serine protease inhibitor comprises the amino acid sequence:
- MAQLCGL RRSRAFLALL GSLLLGVLA -1  
ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
30 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100  
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIIRVARRN 200  
QERALRTVWS SGDDKEQLVK NTYVL 225  
(SEQ ID NO.: 49).
- 35 32. Use according to claim 19, wherein the Kunitz-type serine protease inhibitor comprises the amino acid sequence:

DRAFT  
Version 2  
Page 24  
Page 25

AGSFLAWL GSLLSGVLA - 1  
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF 100  
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
5 ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS 179  
(SEQ ID NO.: 2),

MLR AEADGVSRLL GSLLSGVLA - 1  
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50  
10 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF 100  
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200  
QERALRTVWS SGDDKEQLVK NTYVL 225  
(SEQ ID NO.: 45),

15 MAQLCGL RRSRAFLALL GSLLSGVLA - 1  
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF 100  
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
20 ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200  
QERALRTVWS FGD 213  
(SEQ ID NO.: 47),

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50  
25 YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF 100  
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200  
QERALRTVWS SGDDKEQLVK NTYVL 225  
(SEQ ID NO.: 70),

30 and

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF 100  
35 NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200  
QERALRTVWS FGD 213

(SEQ ID NO.: 71).

33. Use according to claim 19, wherein the Kunitz-type serine protease  
5 inhibitor comprises the amino acid sequence:

IHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKEECLKK CATV 64

(SEQ ID NO.: 4),

10

CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKEECLKK C 61

(SEQ ID NO.: 5),

15

YEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
ACMLRCFRQ 159

(SEQ ID NO.: 6),

20

CTANAVTGPC RASFPRWYFD VERNSCNNFI YGGCRGNKNS YRSEE 150  
ACMLRC 156

(SEQ ID NO.: 7),

25

IHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF 75

NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 125  
ACMLRCFRQ 159

(SEQ ID NO.: 3),

30

CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF 100

NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150  
ACMLRC 156

(SEQ ID NO.: 50),

35

ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 25  
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF 75  
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 125  
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS 179

(SEQ ID NO.: 1),

and

5 ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN  
50  
YLTKKECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF  
100  
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE  
10 150  
ACMLRCFRQQ ENPPLPLGSK 170  
(SEQ ID NO.: 52).

34. Use according to claim 19, wherein the Kunitz-type serine protease  
15 inhibitor comprises the amino acid sequence:

ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN 50  
YLTKKECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DS 92  
(SEQ ID NO.: 8).

20 35. Use according to claim 19, wherein the Kunitz-type serine protease  
inhibitor is glycosylated.

36. Use according to claim 19, wherein the Kunitz-type serine protease  
25 inhibitor contains at least one intra-chain cysteine-cysteine disulfide bond.

37. Use according to claim 19, wherein the Kunitz-type serine protease  
inhibitor contains at least one intra-chain cysteine-cysteine disulfide bond  
selected from the cysteine-cysteine paired groups consisting of CYS11-CYS61,  
30 CYS20-CYS44, CYS36-CYS57, CYS106-CYS156, CYS115-CYS139, and CYS131-  
CYS152, wherein the cysteine residues are numbered according to the amino  
acid sequence of native human placental bikunin.

## ABSTRACT

The instant invention provides for a composition and method for using Kunitz-type serine protease inhibitors, e.g., aprotinin or bikunin, for stimulating the rate of mucociliary clearance of mucus and sputum in lung airways of subjects afflicted with mucociliary dysfunctions such as cystic fibrosis.

**FIGURE 1**

1/48

R35464	GGCCGGGTG	TTCCTCGCCT	GGCTGGGATC	GCTGCTCCTC	TCTGGGTCC	50
ORF	P G R	F S P	G W D R	C S S	L G S	16
R35464	TGGCCGGCCG	ACCGAGAACG	CAGCATCCAC	GACTTCTGCC	TGGTGTGAA	100
ORF	W P A D	R E R	S I H	D F C L	V S K	33
R35464	GGTGGTGGGC	AGATTCCGGG	CCTCCATGCC	TAGGTGGTGG	TACAATGTCA	150
ORF	V V G	R E R A	S M P	R W W	Y N V T	50
R35464	CTGACGGATC	CTGCCAGCTG	TTTGTGTATG	GGGGCTGTGA	CGGAAACAGC	200
ORF	D G S	C Q L	F V Y G	G C D	G N S	66
R35464	AATAATTACC	TGACCAAGGA	GGAGTGCCTC	AAGAAATGTG	CCACTGTAC	250
ORF	N N Y L	T K E	E C L	K K C A	T V T	83
R35464	AGAGAATGCC	ACGGGTGACC	TGGCCACCA	CAGGAATGCA	GC GGATTCC	300
ORF	E N A	T G D L	A T S	R N A	A D S S	100
R35464	CTGTCCCAAG	TGCTCCCAGA	AGGCAGGATT	CTTGAAGACC	ACTTCAGCGA	350
ORF	V P S	A P R	R Q D S	*	R P L Q R	116
R35464	TATGTTCAA	NTATTGNAAG	AATAATTGCA	CCGNCAACGN	ATT-----	393
ORF	Y V S	*	I *	R I I A	P * T *	130

**KEY**

R35464 = Nucleic acid sequence of EST R35464 (SEQ ID NO: 12)  
ORF = EST R35464 Open Reading Frame Translation (SEQ ID NO: 13)

**FIGURE 2**

2/48

R74593	GCAATAATTA	CCTGACCAAG	GAGGAGTGCC	TCAAGAAATG	TGCCACTGTC	50
ORF	Q * L	P D Q G	G V P	Q E M	C H C H	17
R74593	ACAGAGAATG	CCACGGGTGA	CCTGGCCACC	AGCAGGAATG	CAGCGGATTG	100
ORF	R E C H G *	P G H Q	Q E C	S G F		33
R74593	CTCTGTCCCCA	AGTCTCCCAG	AAGGCAGGAT	TCTGAAGACC	ACTCCAGCGA	150
ORF	L C P K S P R	R Q D	S E D H	S S D		50
R74593	TATGTTCAAC	TATGAAGAAT	ACTGCACCGC	CAACGCAGTC	ACTGGGCCTT	200
ORF	M F N Y E E Y	C T A N A V	T G P C			67
R74593	GCCGTGCATC	CTTCCCACGC	TGGTACTTTG	ACGTGGAGAG	GAACTCCTGC	250
ORF	R A S F P R	W Y F D	V E R	N S C		83
R74593	AATAACTTCA	TCTATGGAGG	CTGCCGGGGC	AATAAGAACAA	GCTACCGCTC	300
ORF	N N F I Y G G	C R G	N K N S	Y R S		100
R74593	TGAGGAGGCC	TGCATGCTCC	GCTGCTTCCG	CCAGCAGGAG	AATCCTCCCC	350
ORF	E E A C M L R	C F R	Q Q E	N P P L		117
R74593	TGCCCTTGG	CTCAAAGGTG	GTGGTTCTGG	CCGGGGCTGT	TTCGTGATGG	400
ORF	P L G S K V	V V L A	G A V S	*	W	133
R74593	TGTTGATCCT	TTTCTGGGG	AGCNTCCATG	GTCTTACTGA	TTCCGGGTGG	450
ORF	C * S F S W G	A S M V L L I	P G G			150
R74593	CAAGGAGGAA	CCAGGAGCGT	GCCCTGCGGA	NCGTCTGGAG	CTTCGGAGAT	500
ORF	K E E P G A C	P A X R L E	L R R *			167
R74593	GACAAGGGNT					510
ORF	Q G					169

**KEY**

R74593 = Nucleic acid sequence of EST R74593 (SEQ ID NO: 14)

ORF = EST R74593 Open Reading Frame Translation (SEQ ID NO: 15)

FIGURE 3

3/48

R35464	GGCCGGGTCGT	TTCTCGCCTG	GCTGGGA-TC	GCTGCTCCTC	TCTGGGTCC	50
N39798			TGGGANTC	GCTGCTCCTC	TCTGGGTCC	28
H94519	GCNGCG-CGT	TNNTCGCNT-	GCTGGGA-TC	GCTGCACCTC	TCTGGGTTCG	47
R74593 corr.	-----	-----	-----	-----	-----	-----
Consensus	GGCCGGGTCGT	TTCTCGCCTG	GCTGGGA-TC	GCTGCTCCTC	TCTGGGTCC	50
Translation	A G S F	L A W	L G S	L L L	S G V	-3
R35464	TGGCCGGCCG	ACCGAGAACG	CAGCATCCAC	GACTTCTGCC	TGGTGTGAA	100
N39798	TGG-CGGCCG	ACCGAGAACG	CAGCATCCAC	GACTTCTGCC	TGGTGTGAA	77
H94519	NGG-CGGCCG	ACCGAGAACG	CAGCATCCAC	GACTTCTGCC	TGGTGTGAA	96
R74593 corr.	-----	-----	-----	-----	-----	-----
Consensus	TGG-CGGCCG	ACCGAGAACG	CAGCATCCAC	GACTTCTGCC	TGGTGTGAA	99
Translation	L A A D	B E S	S I H	D E C L	Y S K	15
R35464	GGTGGTGGGC	AGATTCCGGG	CCTCCATGCC	TAGGTGGTGG	TACAATGTCA	150
N39798	GGTGGTGGGC	AGATGCCGGG	CCTCCATGCC	TAGGTGGTGG	TACAATGTCA	127
H94519	GGTGGTGGGC	AGATGCCGGG	CCTCCATGCC	TAGGTGGTGG	TACAATGTCA	146
R74593 corr.	-----	-----	-----	-----	-----	-----
Consensus	GGTGGTGGGC	AGATGCCGGG	CCTCCATGCC	TAGGTGGTGG	TACAATGTCA	149
Translation	V V G	B C B A	S M P	B W W	X N Y I	32
R35464	CTGACGGATC	CTGCCAGCTG	TTTGTGTATG	GGGGCTGTGA	CGGAAACAGC	200
N39798	CTGACGGATC	CTGCCAGCTG	TTTGTGTATG	GGGGCTGTGA	CGGAAACAGC	177
H94519	CTGACGGATC	CTGCCAGCTG	TTTGTGTATG	GGGGCTGTGA	CGGAAACAGC	196
R74593 corr.	-----	-----	-----	-----	GC	2
Consensus	CTGACGGATC	CTGCCAGCTG	TTTGTGTATG	GGGGCTGTGA	CGGAAACAGC	199
Translation	Q G S	C Q L	E Y X G	G C R	G N S	48
R35464	AATAATTACC	TGACCAAGGA	GGAGTGCCTC	AAGAAATGTG	CCACTGTCAC	250
N39798	AATAATTACC	TGACCAAGGA	GGAGTGCCTC	AAGAAATGTG	CCACTGTCAC	227
H94519	AATAATTACC	TGACCAAGGA	GGAGTGCCTC	AAGAAATGTG	CCACTGTCAC	246
R74593 corr.	AATAATTACC	TGACCAAGGA	GGAGTGCCTC	AAGAAATGTG	CCACTGTCAC	52
Consensus	AATAATTACC	TGACCAAGGA	GGAGTGCCTC	AAGAAATGTG	CCACTGTCAC	249
Translation	N N Y L	T K E	E C L	K K C A	T V T	65
R35464	AGAGAATGCC	ACGGGTGACC	TGGCCACCA	CAGGAATGCA	GCGGATTCC	300
N39798	AGAGAATGCC	ACGGGTGACC	TGGCCACCA	CAGGAATGCA	GCGGATTCC	277
H94519	AGAGAATGCC	ACGGGTGACC	TGGCCACCA	CAGGAATGCA	GCGGATTCC	296
R74593 corr.	AGAGAATGCC	ACGGGTGACC	TGGCCACCA	CAGGAATGCA	GCGGATTCC	192
Consensus	AGAGAATGCC	ACGGGTGACC	TGGCCACCA	CAGGAATGCA	GCGGATTCC	299
Translation	E N A	T G D L	A T S	R N A	A D S S	82
R35464	CTGTCCAAG	TGCTCCCAGA	AGGCAGGATT	CTTGAAGACC	ACTTCAGCGA	350
N39798	CTGTCCAAG	TGCTCCCAGA	AGGCAGGATT	CT-GAAGACC	ACTCCAGCGA	326
H94519	CTGTCCAAG	TGCTCCCAGA	AGGCAGGATT	CT-GAAGACC	ACTCCAGCGA	345
R74593 corr.	CTGTCCAAG	TGCTCCCAGA	AGGCAGGATT	CT-GAAGACC	ACTCCAGCGA	151
Consensus	CTGTCCAAG	TGCTCCCAGA	AGGCAGGATT	CT-GAAGACC	ACTCCAGCGA	348
Translation	V P S	A P R	R Q D S	E D H	S S D	98
R35464	TATGTTCAA	NTATTGNAAG	AATAATTGCA	CCGNCAACGN	ATT-----	393
N39798	TATGTTCAA	CTA-TG-AAG	AATACT-GCA	CCGCCAACGC	AGTCACTGGG	372
H94519	TATGTTCAA	CTA-TG-AAG	AATACTGGCA	CCGCCAACGC	ATTCACTGGG	392
R74593 corr.	TATGTTCAA	CTA-TG-AAG	AATACT-GCA	CCGCCAACGC	AGTCACTGGG	197
Consensus	TATGTTCAA	CTA-TG-AAG	AATACT-GCA	CCGCCAACGC	AGTCACTGGG	394
Translation	M F N Y	E E Y C T	A N A V T G	I I I	I I I	113

**FIGURE 3 (CONT)**

4/48

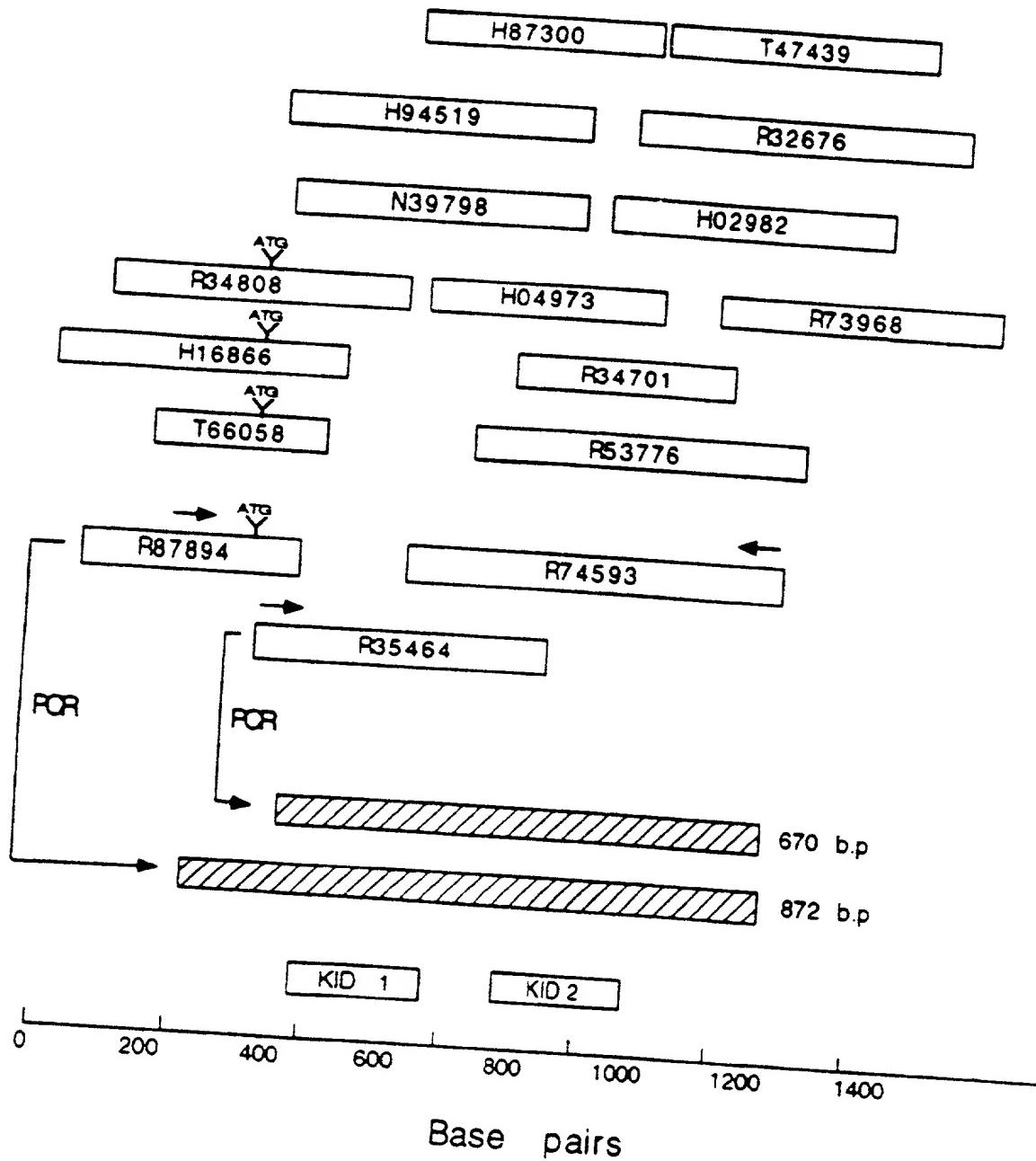
KEY

R35464 = Nucleic acid sequence of EST R35464 (SEQ ID NO.: 12)  
N39798 = Nucleic acid sequence of EST N39798 (SEQ ID NO.: 17)  
H94519 = Nucleic acid sequence of EST H94519 (SEQ ID NO.: 16)  
R74593 corr. = Corrected version of (SEQ ID NO.: 14) G at b.p. 114  
Consensus = Nucleic acid sequence for human bikunin (SEQ ID NO.: 9)  
Translation = Amino acid Translation of Consensus (SEQ ID NO.: 10,

Figure 4 A.

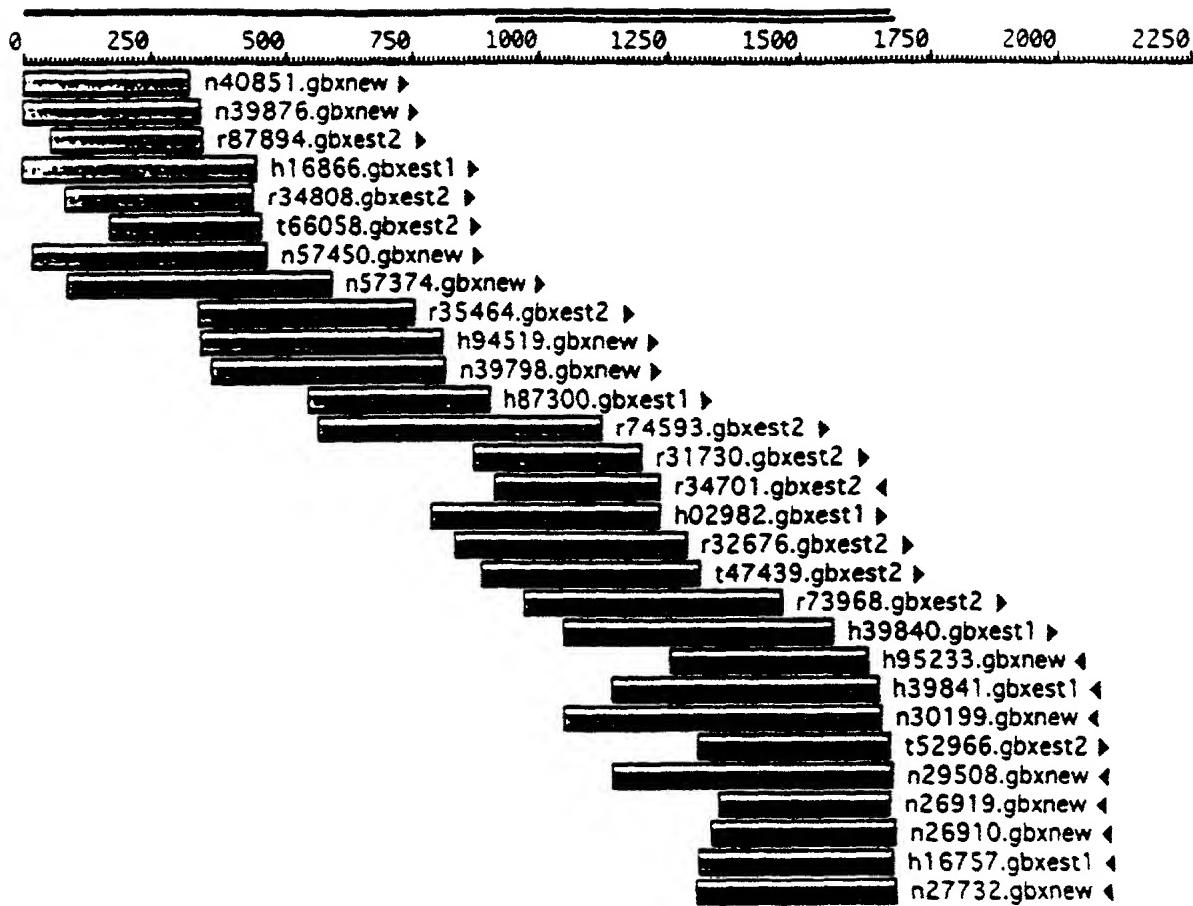
5/48

Schematic depicting the overlap of ESTs bearing homology to the cDNA sequence encoding placental bikinin



6/48

Figure 4B



7/48

Figure 4C

	50
Bikunin	.....GCGA CCTCCGGCGG TTGGGAGGTG TAGCCGGCT CTGAACGGCT
N40851	.....GCGA CCTCCGGCGG TTGGGAGGTG TAGCCGGCT CTGAACGGCT
N39876	.....GCGA CCTCCGGCGG TTGGGAGGTG TAGCCGGCT CTGAACGGCT
R87894	.....
H16866	.....GCCGA CCTCCGGCGG TTGGGAGGTG TAGCCGGCT CTGAACGGGN
R34808	.....
T66058	.....
N57450	..... T TAGCCGGCT CTGAACGCNA
N57374	.....
R35464	.....
H94519	.....
N39798	.....
H87300	.....
R74593	.....
R31730	.....
R34701	.....
HC2982	.....
R32676	.....
T47439	.....
R73968	.....
H39840	.....
H95233	.....
H39841	.....
N30199	.....
T52966	.....
N29508	.....
N26919	.....
N26910	.....
H16757	.....
N27732	.....

8/48

Figure 4C (Con't)

	51	100
Bikunin	GNA GGGCCG TTGACTGTCTG CAGGCAGCGA GGGCGCGACT GAGGAGCAGA	
N40851	NGAGNGGCCG TTGACTGTCTG CAGGCAGCGA GGGCGCGACT GAGGAGCAGA	
N39876	GCA.GGGCCG TTGACTGTCTG CAGGCAGCGA GGGCGCGACT GAGGAGCAGA	
R87894	..... TTGACTGTCTG NAGGCAGCGA GGGCGCGACT GAGGAGCAGA	
H16866	..ANGGGCCG TTGACTGTCTG CAGGCAGCGA GGGCN.GACT GAGGAGCAGA	
R34808	..... .....	G GAGGAGCAGA
T66058	.....	
N57450	GAAGNGGCCG TTGACTGTCTG CAGGCAGCGA GGGCGCGACT GAGGAGCAGA	
N57374	.....	....AGA
R35464	.....	
H94519	.....	
N39798	.....	
H87300	.....	
R74593	.....	
R31730	.....	
R34701	.....	
H02982	.....	
R32676	.....	
T47439	.....	
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
TS2966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

9/48

Figure 4C (Con't)

	101	150
Bikunin	CCCAGGCATC GCGCCGGAG AAGNC GGGC GTCCCCACAC TGAAGGTCCG	
N40851	CCCAGGCATC GCGCCGGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG	
N39876	CCCAGGCATC GCGCCGGAG AAGNC.GGGC NTCCCCACAC TGAAGGTCCG	
R87894	CCCAGGCATC GCGCCGGAG AAGGCCGGGC GTCCCCACAC TGAAGGTCCG	
H16866	CCCAGGCATC GCGCCGGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG	
R34808	CCCAGGCATC GCGCCGGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG	
T66058	.....	
N57450	CCCAGGCATC GCGCCGGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG	
N57374	CCCAGGCATC GCGCCGGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG	
R35464	.....	
H94519	.....	
N39798	.....	
H87300	.....	
R74593	.....	
R31730	.....	
R34701	.....	
H02982	.....	
R32676	.....	
T47439	.....	
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
T52966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

10/48

**Figure 4C (Con't)**

	151	200
Bikunin	GAAAGGGCAC TTCCGGGGGC TTGGCACCT GCGGGACCCCT CCCGGAGCGT	
N40851	GAAAGGGCAC TTCCGGGGGC TTGGCACCT GCGGGACCCCT CCCGGAGCGT	
N39876	GAAAGGGCAC TTCCGGGGGC TTGGCACCT GCGGGACCCCT CCCGGAGCGT	
R87894	GAAAGGGCAC TTCCGGGGGC TTGGCACCT GCGGGACCCCT CCCGGAGCGT	
H16866	GAAAGGGCAC TTCCGGGGGC TTGGCACCT GCGGGACG.T CCCGGAGCG.	
R34808	GAAAGGGCAC TTCCGGGGGC TTGGCACCT GCGGGACCCCT CCCGGAGCGT	
T66058	..... .....	..GGACCCCT CCCGGAGCGT
N57450	GAAAGGGCAC TTCCGGGGGC TTGGCACCT GCGGGACCCCT CCCGGAGCGT	
N57374	GAAAGGGCAC TTCCGGGGGC TTGGCACCT GCGGGACCCCT CCCGGAGCGT	
R35464	..... .....	.....
H94519	..... .....	.....
N39798	..... .....	.....
H87300	..... .....	.....
R74593	..... .....	.....
R31730	..... .....	.....
R34701	..... .....	.....
H02982	..... .....	.....
R32676	..... .....	.....
T47439	..... .....	.....
R73968	..... .....	.....
H39840	..... .....	.....
H95233	..... .....	.....
H39841	..... .....	.....
N30199	..... .....	.....
T52966	..... .....	.....
N29508	..... .....	.....
N26919	..... .....	.....
N26910	..... .....	.....
H16757	..... .....	.....
N27732	..... .....	.....

11/48

Figure 4C (Con't)

	201	250
Bikunin	CGGCACCTGA ACGCGAGGGG CTCCATTGCC CGTGCCTTTC .AGGGGCTTC	
N40851	CGGCACCTGA ACGCGAGGGG CTCCATTGCC CGTGCCTNTG .AGGGGCTTC	
N39876	CGGCACCTGA ACGCGAGGGG CTCCATTGCC CGTGCCTTTC .AGGGGCTTC	
R87894	CGGCACCTGA ACGCGAGGGG CTCCATTGCC CGTGCCTTTC .AGGGGCTTC	
H16866	.GGCACCTGA ACGCGAGGGG CTCCATTGCC CGTGCCTTTC .AGGGGCTTC	
R34808	CGGCACCTGA ACGCGAGGGG CTCCATTGCC CGTGCCTNTG GAGGGGCTTC	
T66058	CGGCACCTGA ACGCGAGGC. CTCCATTGCC .GTGCCTGTC NAGGGGCTTC	
N57450	CGGCACCTGA ACGCGAGGGG CTCCATTGCC CGTGCCTTTC .AGGGGCTTC	
N57374	CGGCACCTGA ACGCGAGGC. CTCCATTGCC. CGTGCCTNG .AGGGGCTTC	
R35464	.....	
H94519	.....	
N39798	.....	
H87300	.....	
R74593	.....	
R31730	.....	
R34701	.....	
HC2982	.....	
R32676	.....	
T47439	.....	
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
T52966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

Figure 4C (Con't)

	251	300
B1kunin	CCGCACCT G ATCCCGAGAC CCCAACGGCT GGTGG CGTC GC TG CGCG	
N40851	CCGCACCT.G ATCCCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG	
N39876	CCGCACCT.G ATCCCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG	
R87894	CCGCACCT.G ATCCCGAGAC CCCAACGGCT GGTNG.CGTC GC.TN.CGCG	
H16866	CCGCACCT.G ATCCCGAGAC CCCAACGGCT GGTNG.CGTC GC.TGGCGCG	
R34808	CCGCACCT.G ATCCCGAGAC CCCAACGGCT GGTGGGGCGT GC.TG.CGCG	
T66058	CCGCACCT.G ATCCCGAGAC CCCAACGGCT GGTGG.CGTC GC.TG.CGCG	
N57450	CCGCACCT.G ATCCCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG	
N57374	CCGGAACTTG ATCCCGAGAC CCCAACGGCT GGTGG.CGTC GC.TG.CGCG	
R35464	.....	
H94519	.....	
N39798	.....	
H87300	.....	
R74593	.....	
R31730	.....	
R34701	.....	
H02982	.....	
R32676	.....	
T47439	.....	
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
T52966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

13/48

Figure 4C (Con't)

	30:	350
Bikunin	TC TCGGCTG AGCT GGCCA TGGCCGANT	GTTGC GGGC T GAGCC GG
N40851	TC TCGGCTG AGCT GGCCA TGTCC	
N39876	TC TCGGCTG AGCT GGCCA TGGCCCACT	G TCGGGNGC T GAGCC GG
R87894	TC TCGGCTG AGCT GGCCA TGGCCGANT	GTTNC GGGC T NAGGC GG
H16866	TTCTCGGCTG AGCT GGCCA TGGCCGANT	GTTGC GNGC T GAGCC GG
R34808	TCTTCGGCTG AGCT GGCCA TGGCCGANT	GTTGC GGGC T GAGCC GG
T66058	TC TCGGCTG AGCT GGCCA TGGCCGANT	GTTGC GNGC T GAGCC GG
N57450	TC TCGGCTG AGCT GGCCA TGGCCGANT	GGTGC GGGC TTGAGGC GG
N57374	TCTTCGGCTG AGCT GGCCA TGGCCGANT	G GTGCCGNGC T GAGCCGG
R35464	.....	..... .GGCCGG
H94519	.....	.....
N39798	.....	.....
H87300	.....	.....
R74593	.....	.....
R31730	.....	.....
R34701	.....	.....
H02982	.....	.....
R32676	.....	.....
T47439	.....	.....
R73968	.....	.....
H39840	.....	.....
H95233	.....	.....
H39841	.....	.....
N30199	.....	.....
T52966	.....	.....
N29508	.....	.....
N26919	.....	.....
N26910	.....	.....
H16757	.....	.....
N27732	.....	.....

**Figure 4C (Con't)**

351	400
B1kunin AC GG CG TTTCTCG CC TGCTGGG A TCGCT GC T CCTCTCT	
R87894 ACG.	
H16866 AC..CGNCGT TTTTCTCG. CCTTGCTGGG ATTGGCTTCG TTCTCTCTG	
R34808 ACCGGGNCG. .TTTTTCGN CCTTGCTGGG ATTCG.TTG. TTNCTCTCTN	
T66058 ...CGGNCG. .TTTCTCG. CC.TGCTGGG A.TCGCT.GC T.CCTCTCT.	
N57450 ANN.NGCCG. ..TTTCTCG. CC.TGCTGGG A.TCGCT.GC T.CCTCTCT.	
N57374 AG..GGCCGG ..TTTCTCG. CCTTGCTGGG A.TCGCT.GC T.CCTCTCTG	
R35464 .....GTCG. ..TTTCTCG. CCTGGCTGGG A.TCGCT.GC T.CCTCTCT.	
H94519 .GCNGCCG. ..TTNNTCG. CN.TGCTGGG A.TCGCT.GC A.CCTCTCT.	
N39798 ..... .... .... CTGGG ANTGGT.GC T.CCTCTCT.	
H87300 .....	
R74593 .....	
R31730 .....	
R34701 .....	
H02982 .....	
R32676 .....	
T47439 .....	
R73968 .....	
H39840 .....	
H95233 .....	
H39841 .....	
N30199 .....	
T52966 .....	
N29508 .....	
N26919 .....	
N26910 .....	
H16757 .....	
N27732 .....	

15/48

Figure 4C (Con't)

	401	450
Bikunin	GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC.ACGACTT.CT	
H16866	GGGGTTCTG GG.CCGCCGA CCGA.GAACG CA.GCA.TCC.AAGAATTCTT	
RJ4808	GGGGTTC.TG GGGNCGCGA NCGA.GAACG CAAGCA.TTC ACGA.TTT	
T66058	GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC.ACGANTT.CT	
N57450	GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC.ACGACTT.CT	
N57374	GGGG.TCCTG G..CGGCCGA NCGAAGAANG CA.GCAATCC ANGAATTNCT	
R35464	GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC.ACGACTT.CT	
H94519	GGGG.TCGNG G..CGGCCGA CCGA.GAACG CA.GCA.TCC.ACGACTT.CT	
N39798	GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC.ACGACTT.CT	
H87300	.....	
R74593	.....	
R31730	.....	
R34701	.....	
H02982	.....	
R32676	.....	
T47439	.....	
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
TS2966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

16/48

Figure 4C (Con't)

	451	500
B1xunin	GCCTGGTGT CGAAGGT GG TGGCCAGATG CCGGG CCTC CATGCCTA G	
H16866	GCC	
T66058	TCCTGGTGT CGAAGG	
N57450	GCCTGGTGT CGAAGGT GG TGGGCAG	
N57374	GCCTGGTGT CGAAAGTTGG TGGGCANATT CCGGGGCCCTT CATGNCTAAC	
R35464	GCCTGGTGT CGAAGGT GG TGGGCAGATT CCGGG CCTC CATGCCTA G	
H94519	GCCTGGTGT CGAAGGT GG TGGGCAGATG CCGGG CCTC CATGCCTA G	
N39798	GCCTGGTGT CGAAGGT GG TGGGCAGATG CCGGG CCTC CATGCCTA G	
H87300	.....	
R74593	.....	
R31730	.....	
R34701	.....	
H02982	.....	
R32676	.....	
T47439	.....	
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
T52966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

17/48

Figure 4C (Con't)

	550
Bikunin	G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT ATG
N57374	GTTGGTGTGGT ANAATGTNAA TTAANGATTC TTGCAACTGT TTGTGTNATT
R35464	G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT ATG
H94519	G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT ATG
N39798	G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT ATG
H87300	.....
R74593	.....
R31730	.....
R34701	.....
H02982	.....
R32676	.....
T47439	.....
R73968	.....
H39840	.....
H95233	.....
H39841	.....
N30199	.....
T52966	.....
N29508	.....
N26919	.....
N26910	.....
H16757	.....
N27732	.....
	551
Bikunin	GGGGCTGTGA CGGAAACA GCAATAATTA CCTGACCAAG GA GGAGTGC
N57374	GGGGCTNTTA AACGGAAANA .CAATAATNA CCTGACCAAA GAAGNAAT..
R35464	GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
H94519	GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
N39798	GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
H87300	GATTCGGCAC AGGGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
R74593	..... GCAATAATTA CCTGACCAAG GA.GGAGTGC
R31730	.....
R34701	.....
H02982	.....
R32676	.....
T47439	.....
R73968	.....
H39840	.....
H95233	.....
H39841	.....
N30199	.....
T52966	.....
N29508	.....
N26919	.....
N26910	.....
H16757	.....
N27732	.....
	600

Figure 4C (Con't)

	601	650
Bikunin	CTCAAGAAAT GTGCCACTGT CACAGAGAAAT GCCACGGGTG ACCTGGCCAC	
RJ5464	CTCAAGAAAT GTGCCACTGT CACAGAGAAAT GCCACGGGTG ACCTGGCCAC	
H94519	CTCAAGAAAT GTGCCACTGT CACAGAGAAAT GCCACGGGTG ACCTGGCCAC	
N39798	CTCAAGAAAT GTGCCACTGT CACAGAGAAAT GCCACGGGTG ACCTGGCCAC	
H87300	CTCAAGAAAT GTGCCACTGT CACAGAGAAAT GCCACGGGTG ACCTGGCCAC	
R74593	CTCAAGAAAT GTGCCACTGT CACAGAGAAAT GCCACGGGTG ACCTGGCCAC	
R31730	.....	
R34701	.....	
H02982	.....	
R32676	.....	
T47439	.....	
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
TS2966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	
	651	700
Bikunin	CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG	
RJ5464	CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG	
H94519	CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG	
N39798	CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG	
H87300	CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG	
R74593	CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGT.CTCCC AGAAGGCAGG	
R31730	.....	
R34701	.....	
H02982	.....	
R32676	.....	
T47439	.....	
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
TS2966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

Bikunin RJ5464 H94519 N39798 H87300 R74593 R31730 R34701 H02982 R32676 T47439 R73968 H39840 H95233 H39841 N30199 TS2966 N29508 N26919 N26910 H16757 N27732

19/48

Figure 4C (Con't)

	701	750
Bikunin	ATTCT GAAG ACCACTCCAG CGATATGTT CAACTAT G AAGAATACTG	
R35464	ATTCTGAAG ACCACTTCAG CGATATGTT CAANTATGNN AAGAATAATT	
H94519	ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG	
N39798	ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG	
H87300	ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG	
R74593	ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG	
R31730	.....	
R34701	.....	
H02982	.....	
R32676	.....	
T47439	.....	
R73968	.....	
H39840	.....	
H95233	.....	
R39841	.....	
N30199	.....	
T52966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	
	751	800
Bikunin	CACCGCCAA CGCACT CAC TGGCC TTG CCGTG CAT CCTT CCCAC	
R35464	GCACCGNCAA CGNATT	
H94519	GCACCGCCAA CGCATT.CAC TGGCC..TG C.GTG.CAT. CCTT.CCCAC	
N39798	.CACCGCCAA CGCACT.CAC TGGGCCCTTG C.GTGGAT. CCTTCCCAC	
H87300	.CACCGCCAA CGCACTNCAC TGGCC..TTG C.GTGGCATN CCTT.CCCAC	
R74593	.CACCGCCAA CGCACT.CAC TGGCC..TTG CCGTG.CAT. CCTT.CCCAC	
R31730	.....	
R34701	.....	
H02982	.....	
R32676	.....	
T47439	.....	
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
T52966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

20/48

Figure 4C (Con't)

	801	850
Bikunin	GCTGGTACTT T GACGTGGA GA GGAACTC CTG CAATAA CTTCATCTAT	
H94519	GCTGGTACTT T.GNCGT	
N39798	GCTGGNAAATT TNGACGTTGA GAAGGAAC	
H87300	GCTTNGTACTT T.GACGTGGA GA.GGAACTC CTGGCAATAA CTTCATCTAT	
R74593	GCTGGTACTT T.GACGTGGA GA.GGAACTC CTG.CAATAA CTTCATCTAT	
R31730	.....	
R34701	.....	
H02982	..... GA GA.GGAACTC CTG.CAATAA CTTCATCTAT	
R32676	.....	G ATTC..GGAA
T47439	.....	
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
T52966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	
	851	900
Bikunin	GGAGGC GC CGGGCCAAT AAGAACAG C TACCGCTC T GAGGAGCCCT	
H87300	GGAGGCTTGC CGGGCCAATN AAGAACAGNT TACCGCTCTT TAGGAGCCCT	
R74593	GGAGGCT.GC CGGGCCAAT. AAGAACAG.C TACCGCTC.T GAGGAGCCCT	
R31730	..... G.C TACCGCTC.T GAGGAGCCCT	
R34701	.....	
H02982	GGNGCCT.GC CGGGG.AAT. AAGAACAC.NC TACCGCTC.T GAGGAGCCCT	
R32676	CGAGGA..GC CGGGCCAAT. AAGAACAG.C TACCGCTC.T GAGGAGCCCT	
T47439	.....	....NGCCCT
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
T52966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

21/48

**Figure 4C (Con't)**

	901	950
Bikunin	GCA TGCTC CGCTGCTTCC CC	CA GCAGGA
H87300	.GCA.T.....	
R74593	.GCA.TGCTC CGCTGCTTCC GC.....	.CA.GCAGGA
R31730	.GCA.TGCTC CGCTGCTTCC GC.....	.CA.GCAGGA
R34701	..... TTCC GC.....	.CAAGCAGGA
H02982	.GCG.TGCTC CGCTGCTTCC GCTGTGTGTT CTCTTCCAGG CCA.GCAGGA	
R32676	.GCA.TGCTC CGCTGCTTCC GC.....	.CA.GCAGGA
T47439	TGCAGTGCTC CGCTGCTTCC GC.....	.CA.GCAGGA
R73968	.....	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
T52966	.....	
H29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	
		1000
Bikunin	GAA TCCTCC CCTCCCCCTT GGCTCAAAGG TGGTGGTTC TGG CGGGGC	
R74593	GAA.TCCTCC CCTCCCCCTT GGCTCAAAGG TGGTGGTTC. TGGCGGGGC	
R31730	GAA.TCCTCC CCTCCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC	
R34701	AAANTCCTCC CCTCCCCCTT GGCTCAAAGG TGGTGGTTC C TGG.CGGGGC	
H02982	GAA.TCCTCC CCTCCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC	
R32676	GAA.TCCTCC CCTCCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC	
T47439	GAA.TCCTCC CCTCCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC	
R73968	.....	CGGGGC
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
T52966	.....	
H29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

22/4/98

**Figure 4C (Con't)**

	1001	1050
Bikunin	TGTT CGTGA TGGTGGTGTAT CC T CTTCC TGGG AGCCT CC ATGGTC	
R74593	TGTT <del>CGTGA</del> TGGTGGTGTAT CCTT..TTCC TGGGGAGCCT CC.ATGGTCT	
R31730	TGTT .CGTGA TGGTGGTGTAT CC.T.CTTCC TGGGGAGCCT CC.ATGGTC.	
R34701	TGTT .CGTGA TGGTGGTGTAT CCCCTCCCTCC CGGG. AGCCT CCCATGGTCC	
H02982	TGTT .CGTGA TGGTGGTGTAT CC.T.CTTCC TGGG. AGCCT CC.ATGGTN.	
R32676	TGTT .CGTGA TGGTGGTGTAT CC.T.CTTCC TGGG. AGCCT CC.ATGGTC.	
T47439	TGTT .CGTGA TGGTGGTGTAT CC.T.CTTCC TGGG. AGCCT CC.ATGGTC.	
R73968	TGTT .CGTGA TGGTGGTGTAT CC.T.CTTCC TGGG. AGCCT CC.ATGGTC.	
H39840	.....	
H95233	.....	
H39841	.....	
N30199	.....	
T52966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	
	1051	1100
Bikunin	TACC TGAT CCGGGTGGCA CGGAGG AAC C AGG AGCG TCCCCCTGGCC	
R74593	TAC ..TGATT CCGGGTGGCA AGGAGG. AAC C. AGG. AGCG TCCCCCTGGCC	
R31730	TACC .TGAT. CCGGGTGGCA CGCAGGGAAC C. AGGGAGCG TCCCCCTGGCC	
R34701	TACCTGAT. CCGGGTGGCA CGGAGG. AAC CCAGG. ANCG TCCCCCTGGCC	
H02982	TACC .TGAT. CCGGGTNGCA CGGAGG. AAC C. AGGGAGCG TCCCCCTGCGN	
R32676	TACC .TGAT. CCGGGTGGCA CGGAGG. AAC C. AGGGAGCG TCCCCCTGGCC	
T47439	TACC .TGAT. CCGGGTNGCA CGGAGG. AAC C. AGG. AGCG TCCCCCTGGCC	
R73968	TACC .TGAT. CCGGGTGGCA CGGAGG. AAC C. AGG. AGCG TCCCCCTGGCC	
H39840	..... .GGG. AAC C. AGG. AGCG TCCCCCTGGCC	
H95233	.....	
H39841	.....	
N30199	..... .GAGGAACC C. ANG. AGCT TCCCCCTGGCC	
T52966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

23/48

**Figure 4C (Con't)**

	1101	1150
Bikunin	ACCG TCT G GAGCTCCGGA GATGACAAGG ACCAGCTGG TGAAGAAC	
R74593	ANCG.TCT.G GAGCTCCGGA GATGACAAGG GNT	
R31730	ACCG.TCTGG GAGCTCCGGA GATGACAAGG GAGCAGCTGG GTGAAGAAC.	
R34701	ACCG.TCT.G GAGCTCCGGA GATGACAAGG .ACCAGCTGG .TGAAGAAC.	
H02982	ACCG.TCTNG GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.	
R32676	ACCG.TCTGG GAGCTCCGGA GATGACAAGG GAGCAGCTGG .TGAAGAAC.	
T47439	ACCG.TCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.	
R73968	ACCG.TCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.	
H39840	ACCGGTCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.	
H95233	.....	
H39841	.....	
N30199	ACCG.TCT.G GAGCTCCGGA GATNACAANG .AGCAGCTGN .TGAAGAAC	
T52966	.....	
N29508	.....	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	
	1151	1200
Bikunin	ACATATGT C CTGT GACCG CCCTGT CGC C AAGAGG A CT GGGAA	
R31730	ACATATGTC CTGTTGACCG NCCTGTTCGC C.AAGAGG.A TTGGGGAA.	
R34701	ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGAA.	
H02982	ACATATGT.C CTGT.GACCG NCCTGTTCGN C.AAGAGG.A CTNGGGAAA	
R32676	ACATATGTC CTGTTGACCG CCCTGTTGCC C.AAGAGGGA NTGGGGAA.	
T47439	ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGAA.	
R73968	ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGAA.	
H39840	ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.NGGAA.	
H95233	.....	
H39841	..... C. CCCTGT.CGC CAAAAGG.A CT.GGGAA.	
N30199	ACATATGT.C CTGT.GACCG CCCTNT.CGC C.AAGAGG.A CT.GGGAAA	
T52966	.....	
N29508	..... CC. CCCTNT.CGC C.AAGAGG.A CT.GGG.AA.	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	

24/48

**Figure 4C (Con't)**

	1201	1250
Bikunin	GGGAGGGG AGACTAT G TGT GA GCT TTTTTT AA A TAGA GG	
R31730	.GGGAGGGGG A	
R34701	.GGGAGGGG AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TA	
H02982	GGGGAGGGG AGATTAT.G. TGTGA.GTT TTTTTT..AA ANTAC	
R32676	GGGGAGGGGG AGANTATTGT TGTGA.GNT TTTTTTAAA ATTAGGAGGG	
T47439	.GGGAGGGG AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG	
R73968	.GGGAGGGG AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG	
H39840	.GGGAGGGG AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG	
H95233	.....	
H39841	.GGGAGGGGA AACNAT.G. TGT.GAACCT TTTTTT.AAA A.TAGA..GG	
N30199	.GGGAGGNG AGACTAT.G. TGT.AA.GCT TTTTTT..AA A.TAGA..GG	
T52966	.....	
N29508	.GGGAGGGG AGACTA..G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	
	1251	1300
Bikunin	GATTGACTC GGATTG A GT GATC A TTAGGG CT GAGGTCTGTT	
RJ2676	GNTTGANTTC GGGNITTTNA GTTGATCCAT TTAGGGGGNT GAG	
T47439	GATTGACTC .GGATTG.A GT.GATC.A. TTAGGG..CT GAGGTCTNTT	
R73968	GATTGACTC .GGATTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT	
H39840	GATTGACTC .GGATTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT	
H95233	..... A. TTAGGG..CT GAGGTCTGTT	
H39841	GATTGACTC .GGATTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT	
N30199	GATTGACTC .GGATTGGA GT.GATC.A. TTAGGG..CT GAGGTCTGTT	
T52966	.....	
N29508	GATTGACTC .GGATTG.A GT.GATCNA. TTAGGG..CT GAGGTCTGTT	
N26919	.....	
N26910	.....	
H16757	.....	
N27732	.....	
	1301	1350
Bikunin	TCTCTGGAG GTAGGACGGC TGCTTCC TG G TC TGGCA GGGATGGG	
T47439	TCTCTNGGAG STAGGACGA	
R73968	TCTCTGGAG GTAGGACGGC TGCTTCC.TG GGTCTTGGCA GGGATGGG	
H39840	TCTCTGGAG GTAGGACGGC TGCTTCC.TG G.TC.TGGCA GGGATGGG.	
H95233	NCTCTGGAG NTAGGACGGC TGCTTCCCTG G.TC.TGGCA GGGATGGG.	
H39841	TCTCTGGAG GTAGGACGGC TGCTTCCCTG G.TC.TGGCA GGGATGGG.	
N30199	TCTCTGGAG GTAGGACGGC TGCTTCC.TG G.TC.TGGCA GGGATGGG.	
T52966	..... ..TC.TGGCA GGGATGGG.	
N29508	TCTCTGGAG GTAGGACGGC TGCTTCA.TG G.TC.TGGCA GGGATGGG	
N26919	.....	
N26910	.....	
H16757	..... G G.TC.TGGCA GGGATGGG.	
N27732	..... CCCTG GGTCTGNCA AGGNATGGG	

25/48

**Figure 4C (Con't)**

	1351	1400
Bikunin	TTTG CTTTG G AAATCCTC T AGGAGGCT CCTCCT CGC ATGG CC TG	
R73968	TTTG.CTTTG GGAAATCCTC TTNGGAGGCT CCTCCTTCCG ATGGCCCTTG	
H39840	TTTG.CTTTG GAGAATCCTC T.ANGAGGCT CCTCCT CGC ATGG CC TG	
H95233	TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT CGC ATGG CC TG	
H39841	TTTG.CTTTG G.AAANCCNC T.AGGAGGCT CCTCCT CGC ATGG CC TG	
N30199	TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCTTCCG ATGG CC TG	
T52966	TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT CGC ATGG CC TG	
N29508	TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT CGC ATGG CC TG	
N26919	..... .... .GAGGCT CCTCCT CGC ATGG CC TG	
N26910	....CTTT GNAAATCCTC T.AGGAGGCT CCTCCT CGC ATGG CC TG	
H16757	TTTGCCTTG G.AAANCCCTC T.AGGAGGCT CCTCCT CGC ATGG CC TG	
N27732	TTTG.CTTTG G.AAATCCTC TTAGGAGGCT CCTCCT CGC ATGG CC TG	
		1401
Bikunin	CAGT CT GG CAGCAG CCC CGAGTTGTTT CC TCGCTG ATC GATTTC	
R73968	CAGT.CTNGG CAGCANCCCC CGAGTTTTTT TCGCTGCTG ATCCGATTTC	
H39840	CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC	
H95233	CAGTTCT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC	
H39841	CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATNTC	
N30199	CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC	
T52966	CAGT.CT.GG CAGCAG..CC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC	
N29508	CAGT.CT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC	
N26919	CAGT.CTGG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ANC.GATTTC	
N26910	CAGT.CT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATCGGATTTC	
H16757	CAGTNCT.GG CAGCAGACCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC	
N27732	CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ANC.GATTTC	
		1451
Bikunin	TTT CCTCCA GGTAG AGT TTTC TTG CTTATGTGA ATTCCATTGC	
R73968	TTTCTCCA GGTAGAATT TTCTTTT	
H39840	TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTGA ATTCCATTGC	
H95233	TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTGA ATTCCATTGC	
H39841	TTT.CCCCA GGTAG..AGT TTTC.TTG. CTTATGTGA ANTCCATTGC	
N30199	TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTGA ATTCCATTGC	
T52966	TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTGA ATTCCATTGC	
N29508	TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTGA ATTCCATTGC	
N26919	TTT.CCNCCA GGTAG..AGT TTTC.TTG. CTTATGTGA ATTCCATTGC	
N26910	TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTGA ATTCCATTGC	
H16757	TTTACCCCCA GGTAG..AGT TTTCCTTGN CTTATGTGA ATTCCATTGC	
N27732	TTT.CCTCCA GGTAG..AGT TTTC.TTG. CTTATGTGA ATTCCATTGC	

26/48

Figure 4C (Con't)

	1550
Bikunin	CTCTTT CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTT GT
H39840	CTCTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTTGT
H95233	CTCTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTT.GT
H39841	CTCTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTT.GT
N30199	CTCTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTT.GT
TS2966	CTCTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTT.GT
N29508	CTCTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTT.GT
N26919	CTCTTT.CN CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTT.GT
N26910	CTCTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTT.GT
H16757	CTCTTTACT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTT.GT
N27732	CTCTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTT.GT
	1551
Bikunin	CTGATTTATG G .TTTTTT AAGTATAAAC AAAAGTTTT TATTAGCATT
H39840	CTGATTTATG GGTTTTTTT AAGTAT
H95233	CTGATTTATG G..TTTTTT AAGTATAAAC AAAAGTTTT TATTAGCATT
H39841	CTGATTTATG G..TTTTTT AAGTATAAAC AAAAGTTTT TATTAGCATT
N30199	CTGATTTATG G..TTTTTT AAGTATAAAC AAAAGTTTT TATTAGCATT
TS2966	CTGATTTATG G..TTTTTT AAGTATAAAC AAAAGTTTT TATTAGCATT
N29508	CTGATTTATG G..TTTTTT AAGTATAAAC AAAAGTTTT TATTAGCATT
N26919	CTGATTTATG G..TTTTTT AAGTNTAAC AAAAGTTTT TATTAGCATT
N26910	CTGATTTATG G..TTTTTT AAGTATAAAC AAAAGTTTT TATTAGCATT
H16757	CTGATTTATG G..TTTTTT AAGTATAAAC AAAAGTTTT TATTAGCATT
N27732	CTGATTTATG G..TTTTTT AAGTATAAAC AAAAGTTTT TATTAGCATT
	1601
Bikunin	CTGAAAAGAAG GAAAGTAAGG TGACAAAGTT TAATAAAAAG GGGCCTTCCC
H95233	CTGAAAAGAAG GAAAGTAAAA TGACAAAGTT TAATAAA
H39841	CTGAAAAGAAG GAAAGTAAGG TGACAAAGTT TAATAAAAAG GGGCCTTCCC
N30199	CTGAAAAGAAG GAAAGTAAAA TGACAAAGTT TAATAAAAAG GGGCCTTCCC
TS2966	CTGAAAAGAAG GAAAGTAAAA TGACAAAGTT TAATAAAAAG GGGCCTTCCC
N29508	CTGAAAAGAAG GAAAGTAAAA TGACAAAGTT TAATAAAAAG GGGCCTTCCC
N26919	CTGAAAAGAAG GAAAGTAAAA TGACAAAGTT TAATAAAAAG GGGCCTTCCC
N26910	CTGAAAAGAAG GAAAGTAAAA TGACAAAGTT TAATAAAAAG GGGCCTTCCC
H16757	CTGAAAAGAAG GAAAGTAAAA TGACAAAGTT TAATAAAAAG GGGCCTTCCC
N27732	CTGAAAAGAAG GAAAGTAAAA TGACAAAGTT TAATAAAAAG GGGCCTTCCC
	1651
Bikunin	CTTAG AAT AAAAAAAAAA AAAAAAAAAA AAAAAAAA
H39841	CTTAA.
N30199	CTTAG.AAT AAA
TS2966	CTTAGGAAT NAAAAAAAAGGAGTG
N29508	CTTAG.AAT AAATTTCAGC ATGTGCTTTC AA
N26919	CTTAG.AAT AAAAAAAA AAAAAAAA A
N26910	CTTAG.AAT AAATTTCAGC ATGTGCTTTC AAAAAAA
H16757	CTTAG.AAT AAAAAAAA AAAAAAAA AAAAAAA
N27732	CTTAG.AAT AAAAAAAA AAAAAAAA AAAAAAAA
	1689

**FIGURE 4D**

27/48

EST consens MLRAEADGVS RLLGSLLL SG VLAADRERSI HDFCLVSKVV GRCRASMP RW 50  
EST consens WYNVTDGSCQ LFVYGGCDGN SNNYLTKEEC LKKCATVTEN ATGDLATSRN 100  
EST consens AADSSVPSAP RRQDSEDHSS DMFNYEEYCT ANAVTGPCRA SFPRWYFDVE 150  
EST consens RNSCNNFIYG GCRGNKNSYR SEEACMLRCF RQQENPPLPL GSKVVVLAGL 200  
EST consens FVMVLILELG ASMVYLIRVA RRNQERALRT VWSSGDDKEQ LVKNTYVL 248

FIGURE 4E

28/48

cDNA  
translationACC 3  
T -47cDNA TGATCGCGAG ACCCCAAACGG CTGGTGGCGT CGCCTGCGCG TCTCGGCTGA 53  
translation . S R D P N G W W R R L R V S A E -30cDNA GCTGGCCATG GCGCAGCTGT GCGGGCTGAG GCGGAGCCGG GCGTTCTCG 103  
translation L A M A Q L C G L R R S R A F L A-13cDNA CCCTGCTGGG ATCGCTGCTC CTCTCTGGGG TCCTGGCGGC CGACCGAGAAA 153  
translation L L G S L L L S G V L A A D R E 4cDNA CGCAGCATCC ACGACTTCTG CCTGGTGTG AAGGTGGTGG GCAGATGCCG 203  
translation R S I H D F C L V S K V V G R C R 21cDNA GGCCTCCATG CCTAGGTGGT GGTACAATGT CACTGACGGA TCCTGCCAGC 253  
translation A S M P R W W Y N V T D G S C Q L 38cDNA TGTTTGTGTA TGGGGCTGT GACGGAAACA GCAATAATT A CCTGACCAAG 303  
translation F V Y G G C D G N S N N Y L T K 54cDNA GAGGAGTGCC TCAAGAAATG TGCCACTGTC ACAGAGAATG CCACGGGTGA 353  
translation E E C L K K C A T V T E N A T G D 71cDNA CCTGGCCACC AGCAGGAATG CAGCGGATTG CTCTGCTCCA AGTGCTCCC 403  
translation L A T S R N A A D S S V P S A P R 88cDNA GAAGGCAGGA TTCTGAAGAC CACTCCAGCG ATATGTTCAA CTATGAAGAA 453  
translation R Q D S E D H S S D M F N Y E E 104cDNA TACTGCACCG CCAACGCAGT CACTGGGCCT TGCCGTGCAT CCTTCCCACG 503  
translation Y C T A N A V T G P C R A S F P R 121cDNA CTGGTACTTT GACGTGGAGA GGAACCTCTG CAATAACTTC ATCTATGGAG 553  
translation W Y F D V E R N S C N N F I Y G G 138cDNA GCTGCCGGGG CAATAAGAAC AGCTACCGCT CTGAGGAGGC CTGCATGCTC 603  
translation C R G N K N S Y R S E E A C M L 154cDNA CGCTGCTTCC GCCAGCAGGA GAATCCTCCC CTGCCCTTG GCTCAAAGGT 653  
translation R C F R Q Q E N P P L P L G S K Y 171cDNA GGTGGTTCTG GCGGGCTGT TCGTGATGGT GTTGATCCTC TTCCTGGGAG 703  
translation V V L A G L F V M V L I L F L G A 188cDNA CCTCCATGGT CTACCTGATC CGGGTGGCAC GGAGGAACCA GGAGCGTGCC 753  
translation S M V Y I I R V A R R N Q E R A 204cDNA CTGCGCACCG TCTGGAGCTT CGGAGATGA 732  
translation L R T V W S F G D 213

FIGURE 4F

29/48

CDNA            GCACGAGTTG GGAGGTGAG CGCGGCTCTG AACGCCTGA GGGCCGTTGA 50  
 CDNA            GTGTCGCAGG CGCGGAGGGC GCGAGTGAGG AGCAGACCCA GGCATCGCGC 100  
 CDNA            GCCGAGAAGG CCGGGCGTCC CCACACTGAA GGTCCGGAAA GCGGACTTCC 150  
 CDNA            GGGGGCTTTG GCACCTGGCG GACCCCTCCCG GAGCGTCGGC ACCTGAAACGC 200  
 CDNA            GAGGCCTCC  ATTGCGCGTG CGCGTTGAGG GGCTTCCCGC ACCTGATCGC 250  
 CDNA            GAGACCCCCA CGGCTGGTGG CGTCGCCTGC GCGTCTCGGC TGAGCTGGCC 300  
 CDNA            ATGGCGCAGC TGTGCGGGCT GAGGCGGAGC CGGGCGTTTC TCGCCCTGCT 350  
 translation M A Q L C G L R R S R A F L A L L -11

CDNA            GGGATCGCTG CTCCCTCTCTG GGGTCTCTGGC GGCGGACCGA GAACGCAGCA 400  
 translation G S L L L S G V L A A D R E R S I 7

CDNA            TCCACGACTT CTGCCCTGGTG TCGAAGGTGG TGGGCAGATG CCGGGCCTCC 450  
 translation H D F C L V S K V V G R C R A S 23

CDNA            ATGCCCTAGGT GGTGGTACAA TGTCACTGAC GGATCCCTGCC AGCTGTTGT 500  
 translation M P R W W Y N V T D G S C Q L F V 40

CDNA            GTATGGGGGC TGTGACGGAA ACAGCAATAA TTACCTGACC AAGGAGGAGT 550  
 translation Y G G C D G N S N N Y L T K E E C 57

CDNA            GCCTCAAGAA ATGTGCCACT GTCACAGAGA ATGCCACGGG TGACCTGGCC 600  
 translation L K K C A T V T E N A T G D L A 73

CDNA            ACCAGCAGGA ATGCAGCGGA TTCCCTCTGTC CCAAGTGCTC CCAGAAGGCA 650  
 translation T S R N A A D S S V P S A P R R Q 90

CDNA            GGATTCTGAA GACCACTCCA GCGATATGTT CAACTATGAA GAATACTGCA 700  
 translation D S E D H S S D M F N Y E E Y C T 107

CDNA            CCGCCAACGC AGTCACTGGG CCTTGCCGTG CATCCTTCCC ACGCTGGTAC 750  
 translation A N A V T G P C R A S F P R W Y 123

CDNA            TTTGACGTGG AGAGGAACCTC CTGCAATAAC TTCATCTATG GAGGCTGCCG 800  
 translation F D V E R N S C N N F I Y G G C R 140

CDNA            GGGCAATAAG AACAGCTACC GCTCTGAGGA GGCCTGCATG CTCCGCTGCT 850  
 translation G N K N S Y R S E E A C M L R C F 157

CDNA            TCCGCCAGCA GGAGAACCT CCCCTGCCCT TTGGCTCAAA GGTGGTGGTT 900  
 translation R Q Q E N P P L P L G S K V V V 173

CDNA            CTGGCGGGGC TGTTCTGAT GGTGTTGATC CTCTTCTGG GAGCCTCCAT 950  
 translation L A G L F V M Y I I F L G A S M 190

CDNA            GGTCTACCTG ATCCGGGTGG CACGGAGGAA CCAGGAGCGT GCCCTGCGCA 1000  
 translation V X L I R V A R R N Q E R A L R T 207

CDNA            CCGTCTGGAG CTCCGGAGAT GACAAGGAGC AGCTGGTCAA AAACACATAT 1050  
 translation V W S S G D D K E Q L V K N T Y 223

CDNA            GTCCCTGTGAC CGCCCTGTGCG CCAAGAGGAC TGGGAAGGG AGGGGAGACT 1100  
 translation V L \* 225

**FIGURE 4F (Con't)**

30/48

CDNA	ATGTGTGAGC	TTTTTTAAA	TAGAGGGATT	GACTCGGATT	TGAGTGATCA	1150
CDNA	TTAGGGCTGA	GGTCTGTTTC	TCTGGGAGGT	AGGACGGCTG	CTTCCTGGTC	1200
CDNA	TGGCAGGGAT	GGGTTTGCTT	TGAAATCCCT	CTAGGAGGCT	CCTCCTCGCA	1250
CDNA	TGGCCTGCAG	TCTGGCAGCA	GCCCCGAGTT	GTTTCTCGC	TGATCGATT	1300
CDNA	CTTCCTCCA	GGTAGAGTTT	TCTTGCTTA	TGTTGAATT	CATTGCCTCC	1350
CDNA	TTTTCTCNAT	CACAGAAGTG	ATGTTGGAAT	CGTTTCTTT	GTTTGTCTGA	1400
CDNA	TTTATGGTTT	TTTTAAGTAT	AAACAAAAAGT	TTTTTATTAG	CATTCTGAAA	1450
CDNA	GAAGGAAAGT	AAAATGTACA	AGTTTAATAA	AAAGGGCCT	TCCCCTTTAG	1500
CDNA	1550	AATAAATTTC	CAGCATGTTG	CTTCAAAAAA	AAAAAAAAAA	AAAA

**FIGURE 4G**

31/48

EST consens	MLR AEADGVSRLL GSLLLGVLA	-1
PCR clone	MAQLCGL RRSRAFLALL GSLLLGVLA	-1
λcDNA clone	MAQLCGL RRSRAFLALL GSLLLGVLA	-1
EST consens	ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN	50
PCR clone	ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN	50
λcDNA clone	ADRERSIHDF CLVSKVVGRC RASMPRWYN VTDGSCQLFV YGGCDGNSNN	50
EST consens	YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF	100
PCR clone	YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF	100
λcDNA clone	YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSPRRQ DSEDHSSDMF	100
EST consens	NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE	150
PCR clone	NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE	150
λcDNA clone	NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE	150
EST consens	ACMLRCFRQQ ENPPLPLGSK <u>VVLAGLFVM VLILELGASM VYLIRVARRN</u>	200
PCR clone	ACMLRCFRQQ ENPPLPLGSK <u>VVLAGLFVM VLILELGASM VYLIRVARRN</u>	200
λcDNA clone	ACMLRCFRQQ ENPPLPLGSK <u>VVLAGLFVM VLILELGASM VYLIRVARRN</u>	200
EST consens	QERALRTVWS SGDDKEQLVK NTYVL	225
PCR clone	QERALRTVWS FGD	213
λcDNA clone	QERALRTVWS SGDDKEQLVK NTYVL	225

3248

Purification of Placental Bikunin using Superdex 75 Gel-Filtration

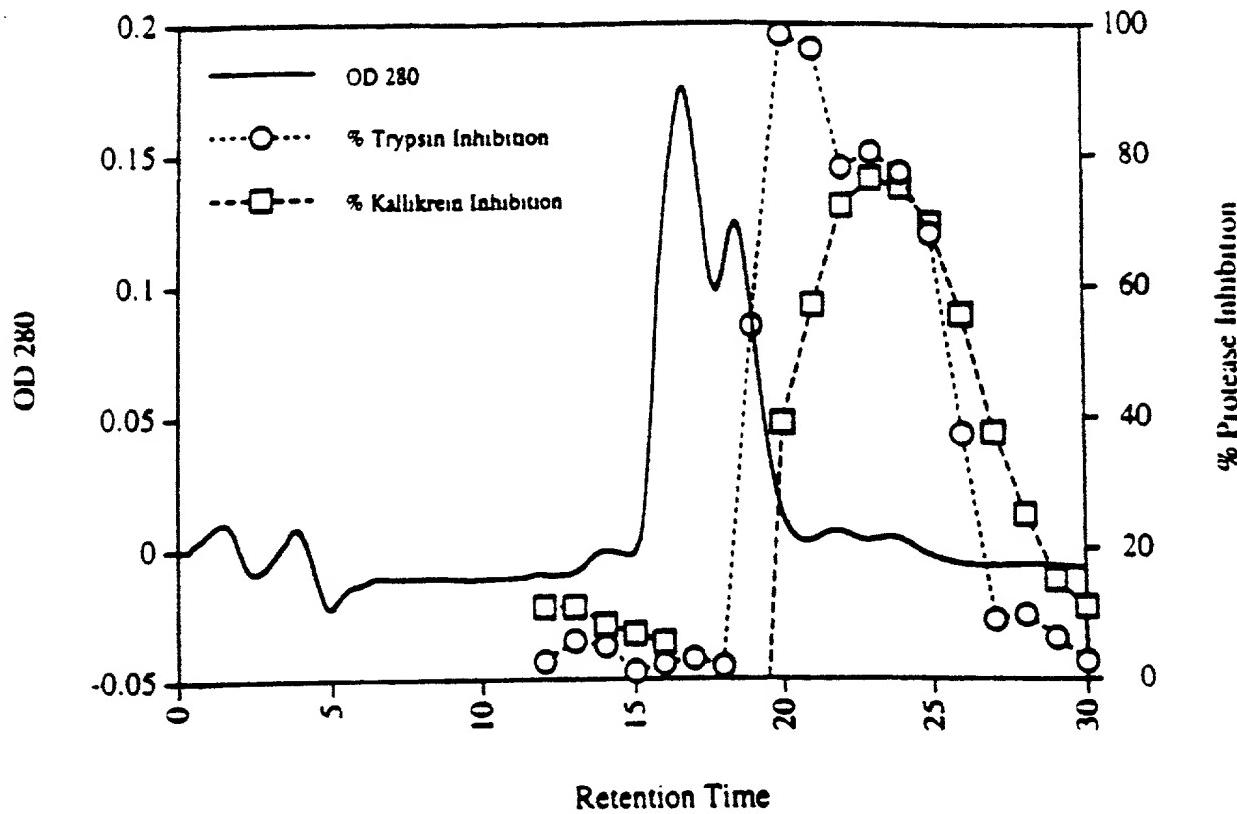
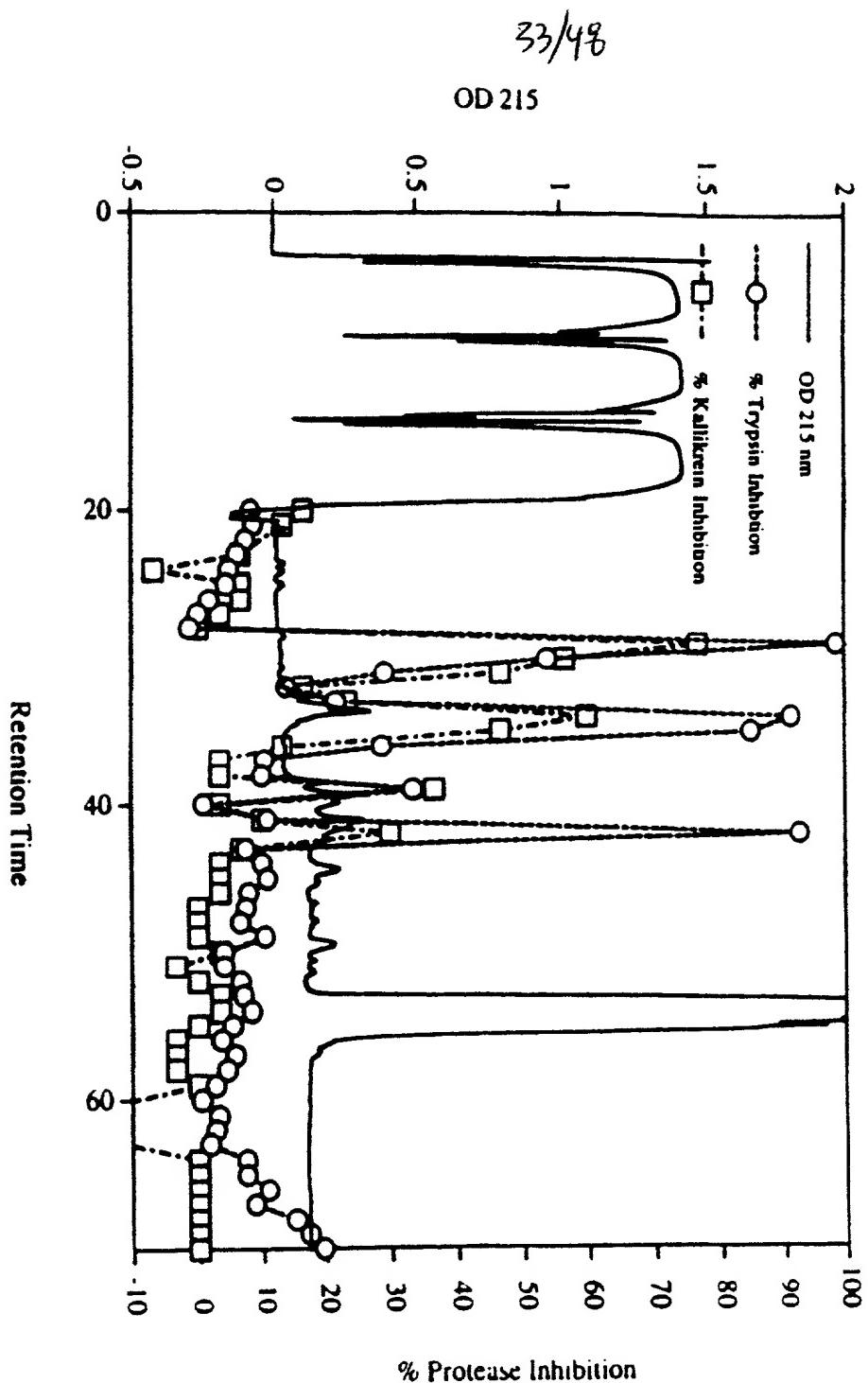


FIGURE 5

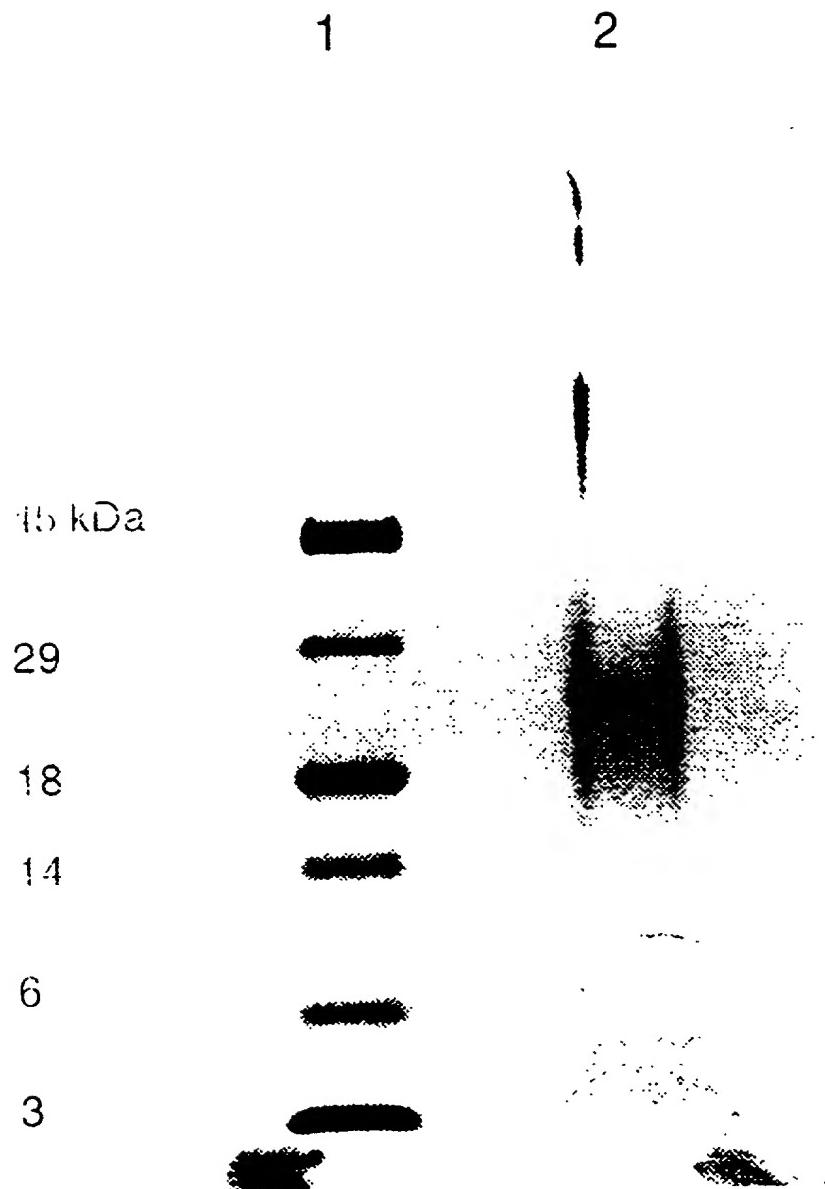
**FIGURE 6**

Purification of Placental Bikunin using C18 Reverse-Phase Chromatography



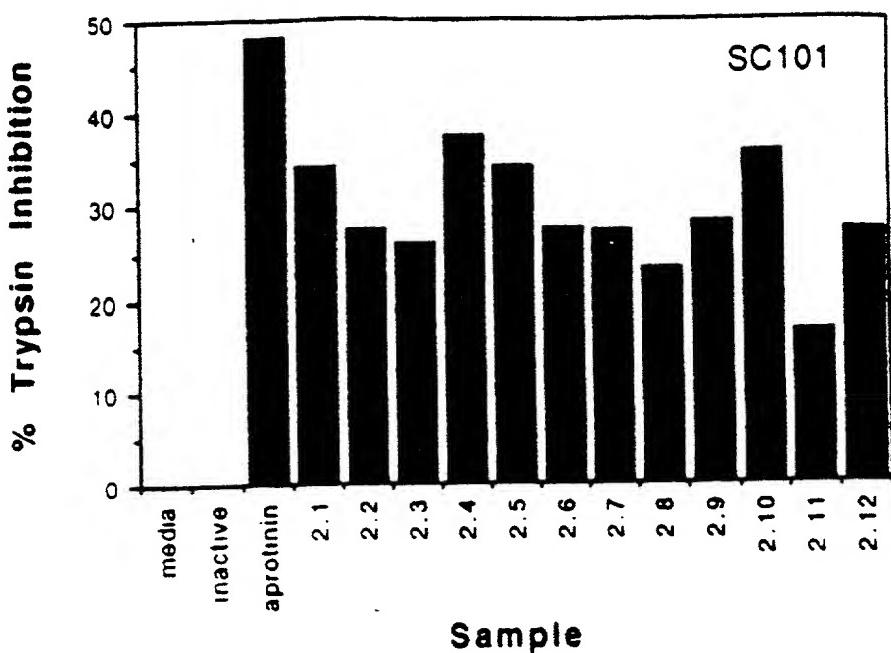
34/48

**Figure 7**

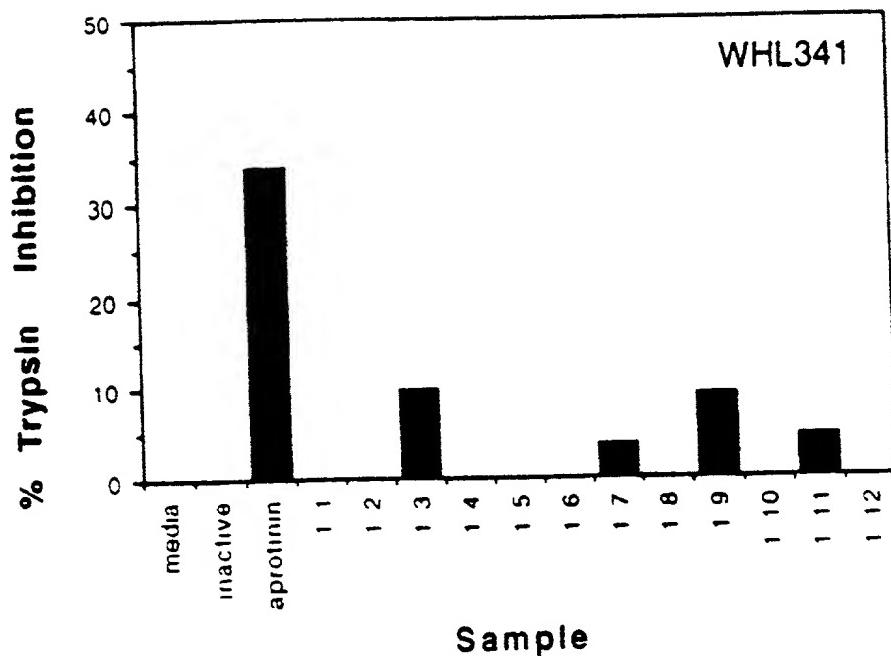


**Figure 8A**

35/48



**Figure 8B**



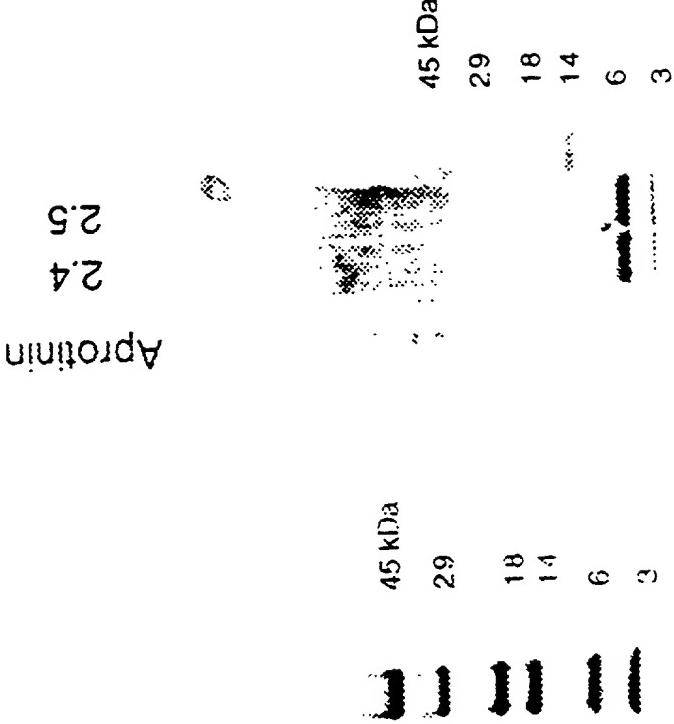
**Figure 9A**

SDS-PAGE

Adrotinin  
2.5  
2.4

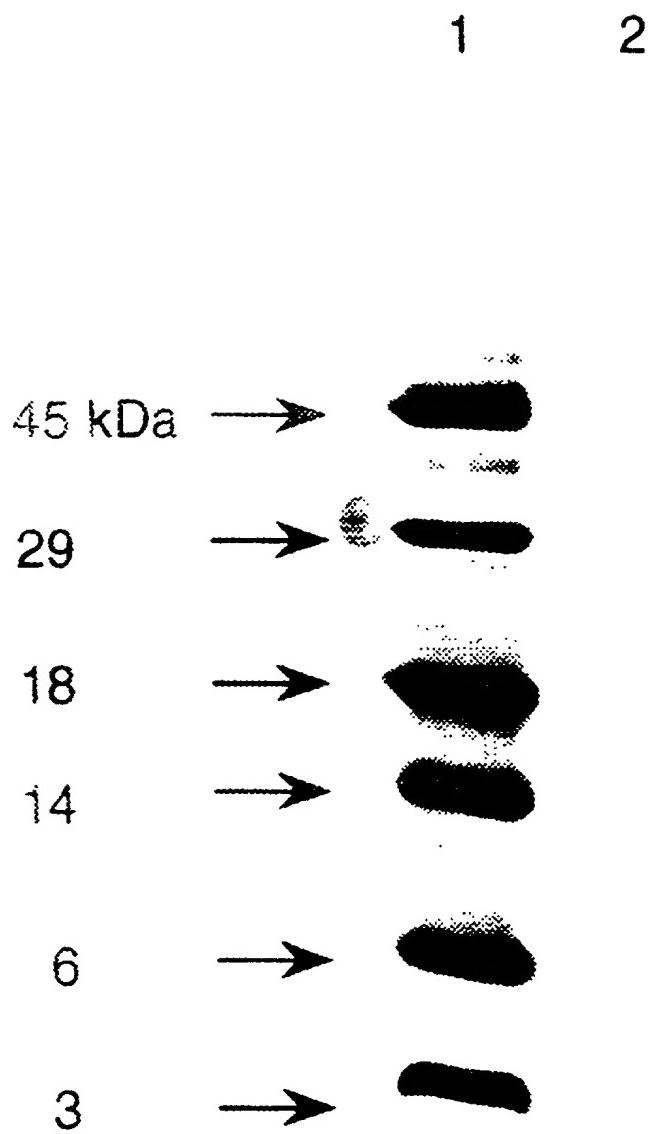
**Figure 9B**

Western

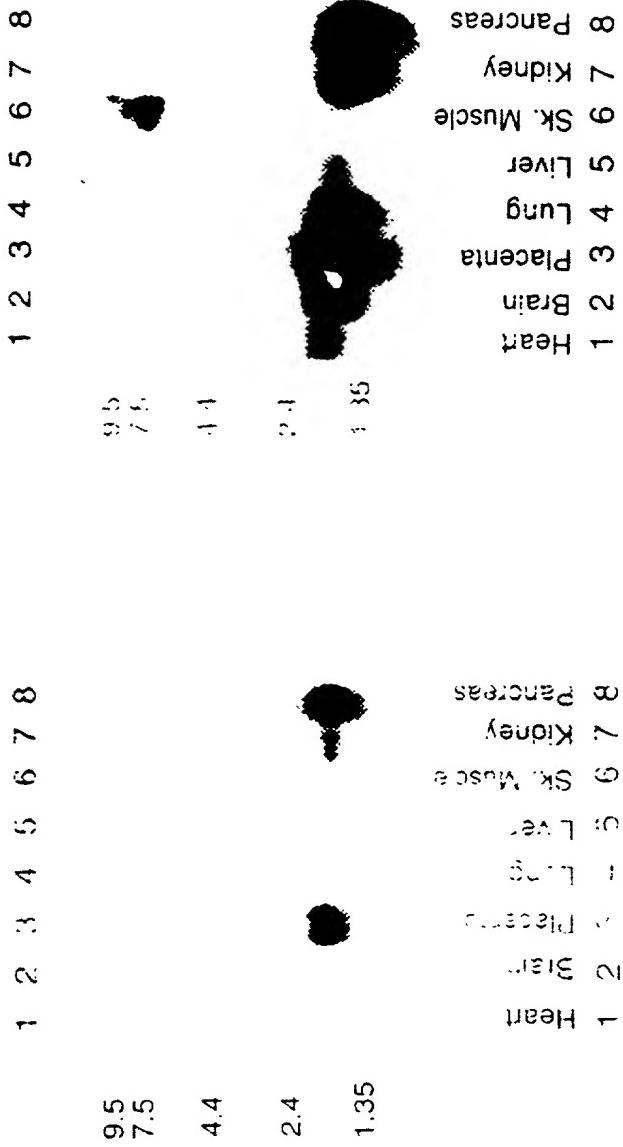


37/48

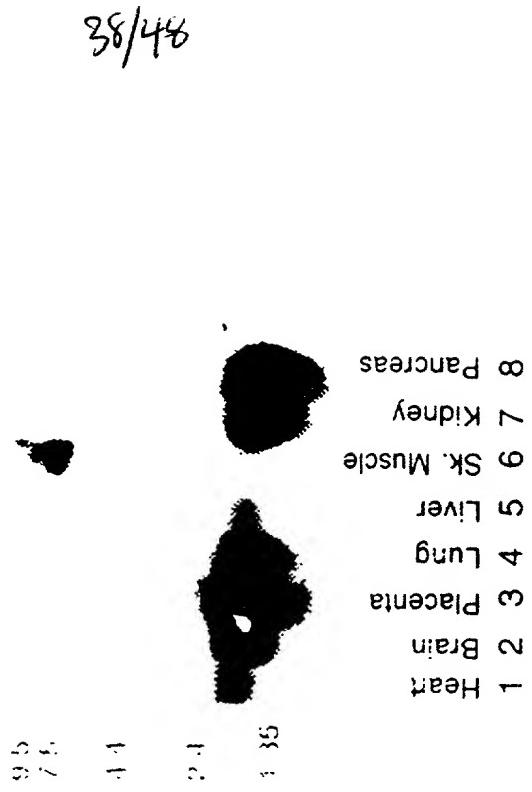
Figure 10



**Figure 11A**



**Figure 11B**



38/48

Figure 12A

1    2    3    4

45 kDa  
29  
18  
14  
6  
3



Figure 12B

1    2    3    4

45 kDa  
25  
18  
14  
6  
3

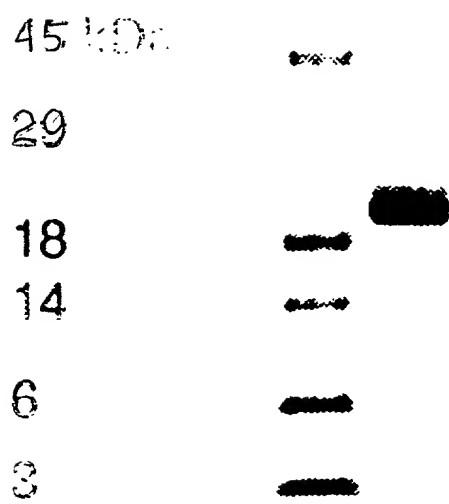


39/48

40/48

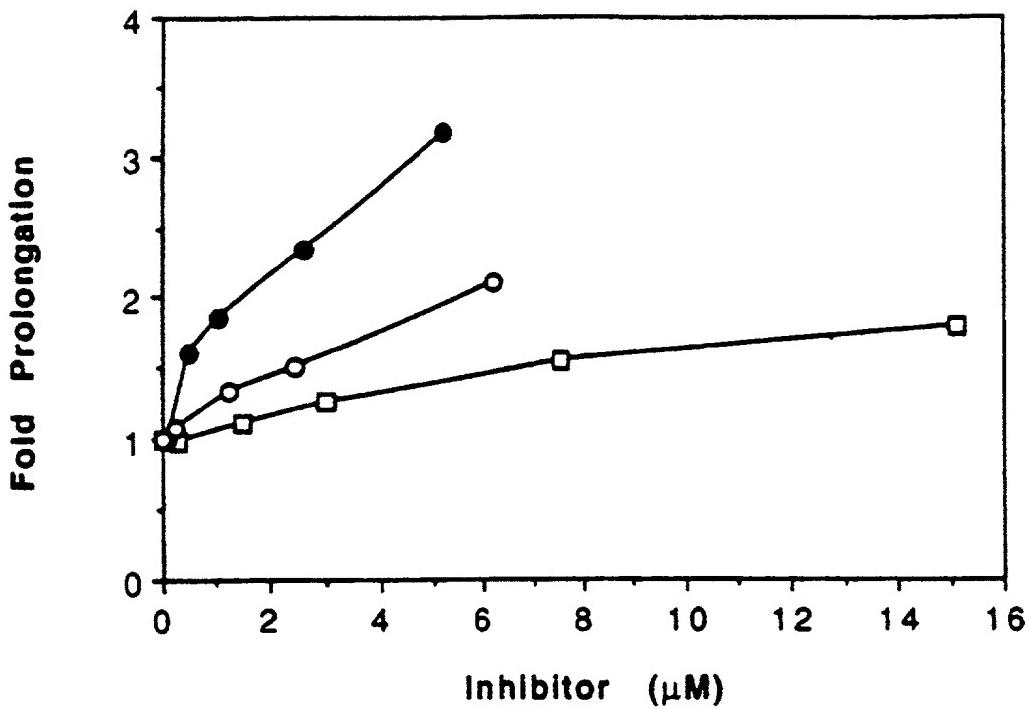
Figure 13

1 2

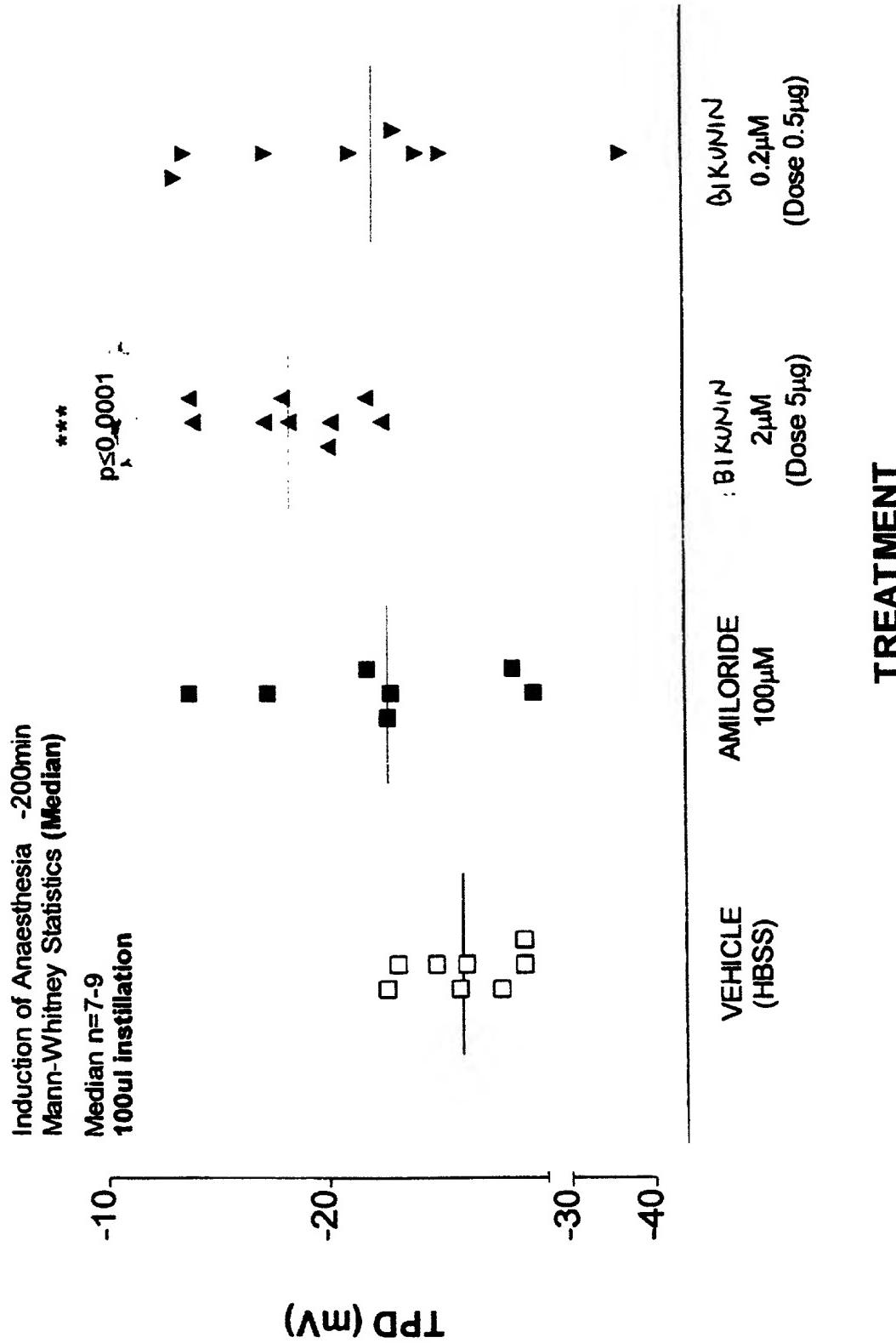


41/48

**Figure 14**

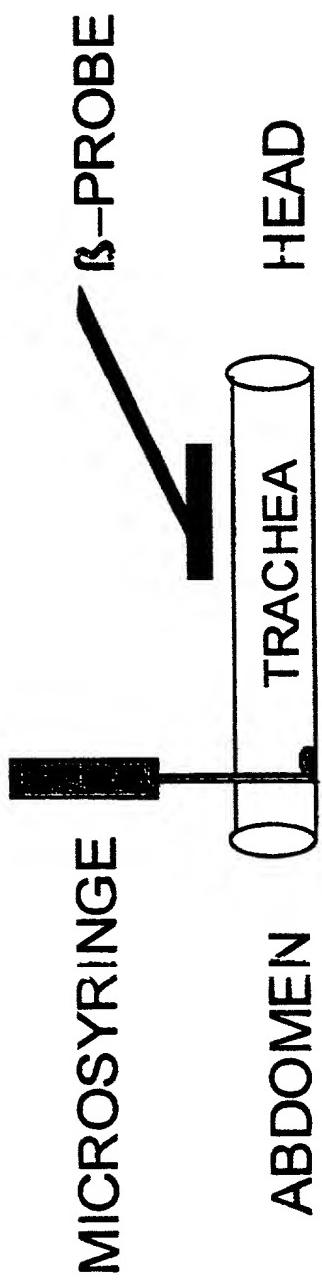


*Figure 15 : The effect of BIKUNIN (0.2-2 $\mu$ M) and amiloride (100 $\mu$ M) on tracheal potential difference (TPD) 3 hours post treatment*



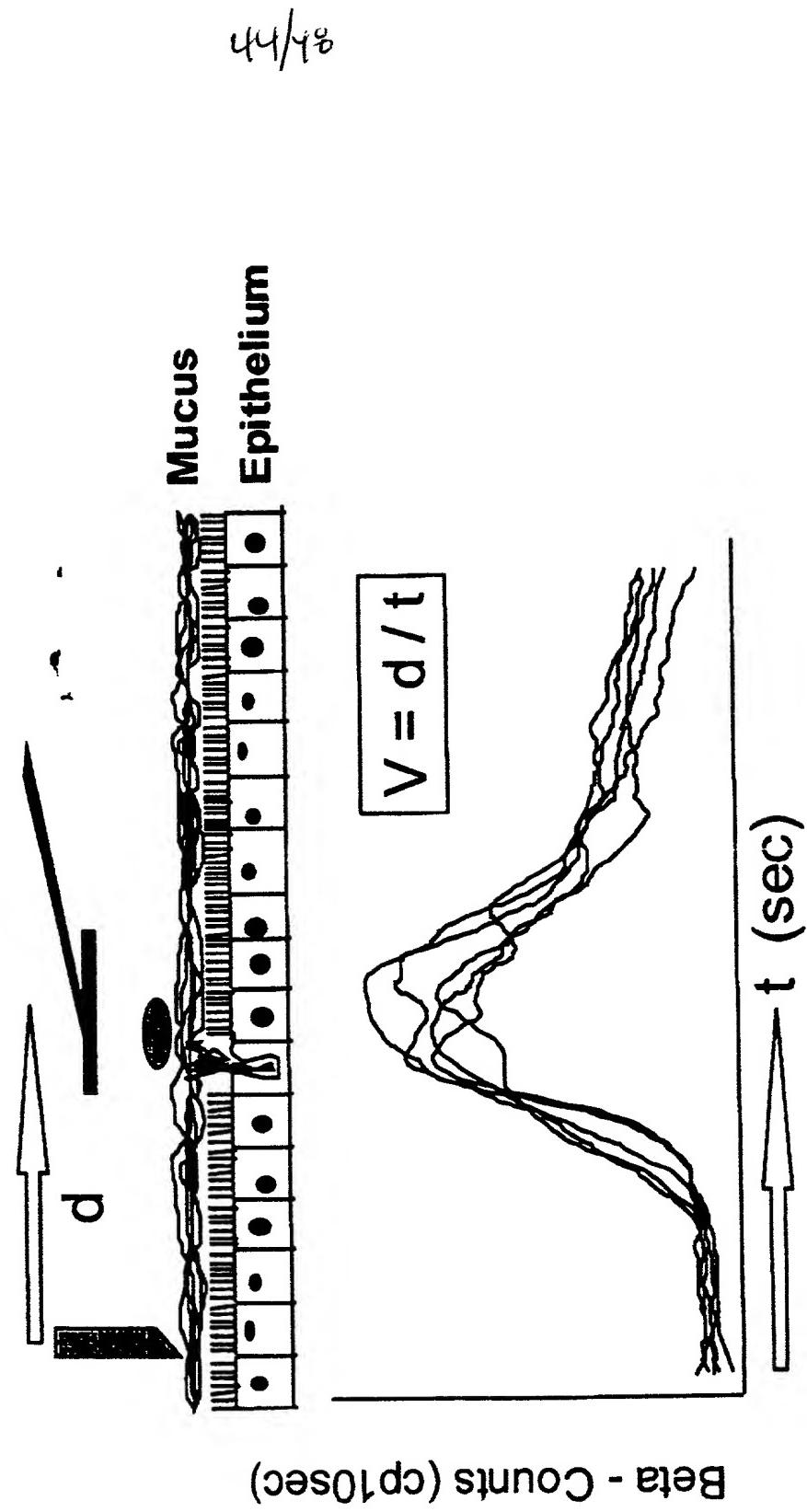
43/48

Figure 16 (a) : Diagram to show the arrangement of needle and Beta probe.

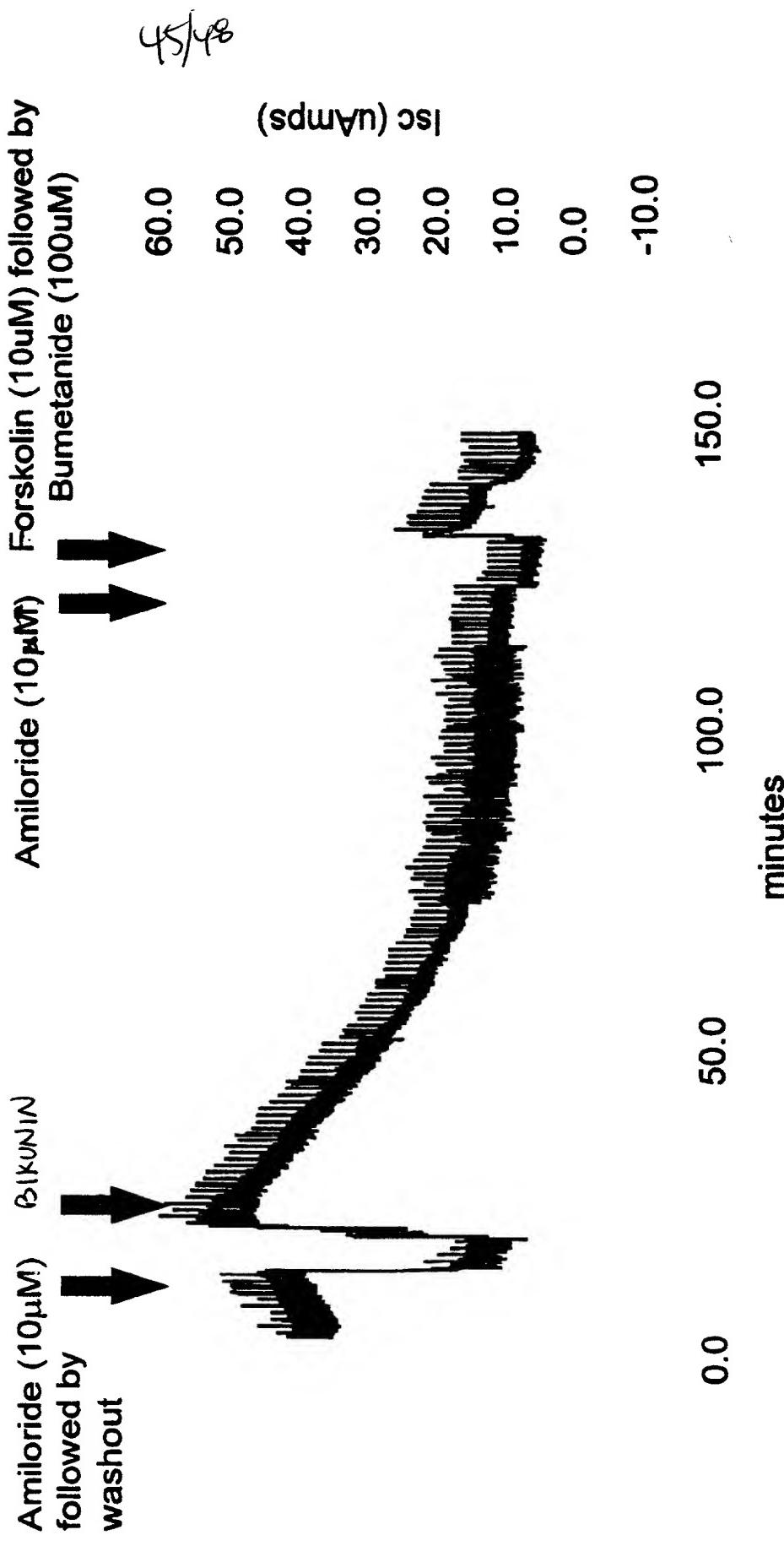


Longitudinal view

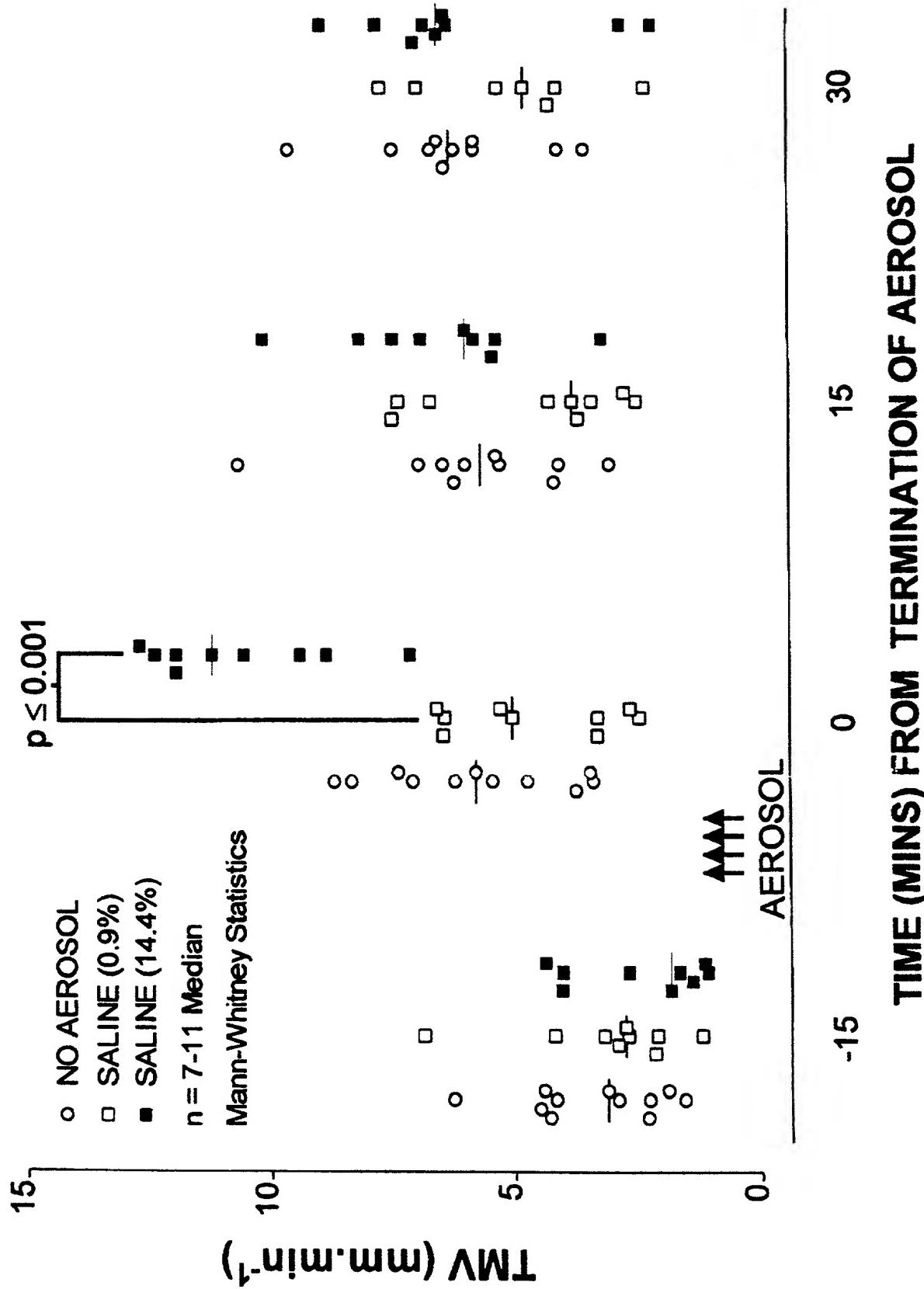
Figure 16 (b) : Counts detected by the probe as the  $^{32}\text{P}$ -labeled *Saccharomyces cerevisiae* are transported along the tracheal mucociliary layer.



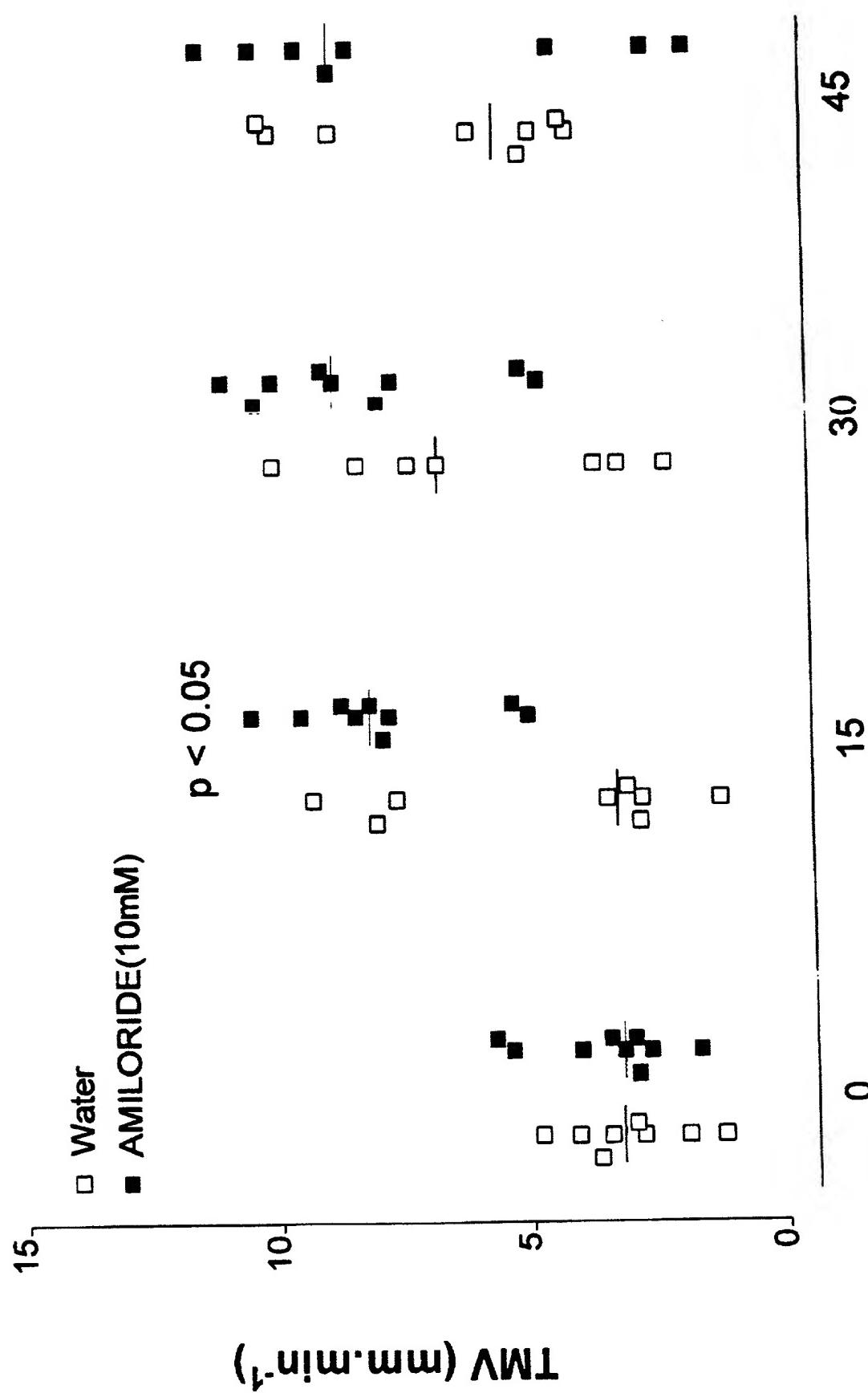
**Figure 17 :** Short circuit current ( $I_{sc}$ ) trace to show the action of  $\beta$ -IKUNIN (70nM) on sodium dependent current in cultured normal human bronchial epithelial cells in vitro



*Figure 18 : The effect of a 5 min aerosol of hypertonic saline (14.4%) on tracheal mucus velocity (TMV) in the anaesthetised spontaneously breathing guinea-pig*



**Figure 19 : The effect of a 20 min aerosol of amiloride (10mM) on tracheal mucus velocity (TMV) in the anaesthetised spontaneously breathing guinea-pig**



49/48

Figure 20 : Short circuit current (Isc) trace to show the action of Aprotinin Dose  
mrtin (0.5-5ug.ml<sup>-1</sup>) on sodium dependent current in normal human  
bronchial epithelial cells in vitro

